In vitro morphogenetic studies in callus cultures of *Medicago sativa* L.

THESIS

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BY

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(M.Sc. BOTANY)

UNDER THE GUIDANCE AND SUPERVISION OF

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Certificate

This is to certified that the thesis entitled "*In vitro* morphogenetic studies in callus cultures of *Medicago sativa* L.", is an original piece of work done by Shalini Jain, M.Sc. (Botany) under my supervision and guidance for the degree of Doctor of Philosophy in Botany, Bundelkhand University, Jhansi.

I, further certify that:

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DECLARATION

I, hereby, declare that the thesis entitled "*In vitro* morphogenetic studies in callus cultures of *Medicago sativa* L.", submitted by me for the award of degree of Doctor of Philosophy in Botany, Faculty of Science, Bundelkhand University, Jhansi (U.P.) is an original piece of research work done by me under the supervision of Dr. M.G. Gupta, Principal Scientist, I.G.F.A.R.I., Jhansi and to the best of my knowledge any part or whole of this thesis work has not been submitted for any degree or any other qualification of any University or examining body in India or elsewhere.

Dated
January 22nd, 2003

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January 22nd, 2003

(SHALINI JAIN)

CONTENTS

List of Table

List of figures

List of abbreviations used in the text and their expansion

2,4-D	2,4-dichlorophenoxy acetic acid
NAA	Naphthalene acetic acid
IAA	Indole 3-Acetic acid
BAP	6- Benzyl amino purine
Kin	Kinetin
CP	Crude Protein
NDF	Neutral detergent fibre
ADF	Acid detergent fibre
AI	Availability index
EE	Ether extract
NDS	Neutral detergent solution
CF	Crude fibre

INTRODUCTION

1. *Introduction*

Medicago sativa L. belongs to family *Fabaceae* and is commonly known as alfalfa or lucerne. The genus *Medicago* has a wide distribution and comprises of more than 50 species exhibiting both annual and perennial habits. Various species of the genus are represented by several chromosomal races ranging from diploids (2n=14 and 16), the tetraploids (2n=32) to hexaploids (2n=48) (Stansford *et al.*, 1972). From the extensive phylogenetic studies of alfalfa and other legumes, Sinskaya (1950) concluded that alfalfa had two centers of origin, the first was the mountainous region of Transcaucasia which gave rise to modern European alfalfa, and the second and independent centre of origin was central Asia. The geographic centre most often mentioned as the homeland of alfalfa is Iran. It is believed that alfalfa evolved in an area with a strong continental climate with cold winters; short, hot and dry summers: late spring; well drained soils of near neutral pH and high lime content (Klinkowski, 1933 and Sinskaya, 1950)

1.1. Types and Varieties:

A workable classification of types and varieties provided by Whyte *et al.* (1953) suggested four groups based on flower colour, origin, winter hardiness, disease resistance and certain agronomic characteristics. (a) The common group - consists of pure *M. sativa* with purple flowers and limited hardiness. (b) The Turkistan group - This also consists of *M. sativa* types with purple flowers. Growth habit is shorter and more spreading than common group. (c) The varigated group - Strains of this group have varigated flower color and are thought to have originated as hybrids between *M. falcata* and *M. sativa*. Varieties are generally winter hardy. (d) The nonhardy group - It is adapted for short days and a long growing season, characterized by an erect habit, quick recovery after cutting susceptibility cold injury and disease.

Purple flowered, common alfalfa (*M. sativa* L.) evolved in dry areas where irrigation provided selection pressure for resistance to diseases and insects. *M. falcata* L. played an important role in evolution of common alfalfa (*M. sativa*). The

introgression of many local endemic ecotypes of *M. falcata* played major role in enriching gene pool for natural selection under a great variety of edaphic and climatic conditions.

1.2. Scientific and Vernacular names:

The most common account of derivation of scientific and common names of alfalfa has been given by Seofield (1908). Apparently the earliest botanists accepted the common Latin name *Medica.* The noted French botanist Tournefort (1700) described alfalfa and its related forms under the group named *Media.* Linnaeus (1735) applied the name *Medica* to alfalfa and its related species and in 1753 in his *species plantanum*, Linnaeus renamed alfalfa as *Medicago sativa.* Alfalfa has been called by many common names throughout its long history. Ancient Greeks called it "Medicai" and Romans "Medica". In Italy it is still known as "erba medica". A very early reference was discovered in a Babilonian text written *Ca* 700 B.C. in which alfalfa was listed under the Iranian name "aspasti". Aspasti can be traced to old Iranian word aspo-asti ("horse-fodder"). Since the Arabs probably obtained alfalfa from Persia (Iran) it appears that they modified aspasti through various stages to alfalfa.

There is some what more controversy over the derivation of the term lucerne (spelled also as lucern, luserne and luzern). Piper (1935) claimed that the word lucerne was first used in 1587. However, Hendry (1923) and most other authorities including Bolton (1962) agree that alfalfa came into general culture in the Lucerne Lake region of Switzerland as early as 1st century, and in its subsequent dispersion through Europe it was known as lucerne. It might have also have been named from the Lucerna river valley in Italy, in principel where it is now cultivated, is known either as "alfalfa" or "Lucerne".

Lucerne, also known as "the queen of the forages", is one of the most important cultivated leguminous forage crop and is grown on an estimated area of more than 33 million hectares in diverse environmental conditions throughout the world with

its distribution ranging from temperate to equatorial regions (Bolton *et al.*, 1972). It is the only forage known to have been cultivated before the era of recorded history. In near Eastern region, it has been grown for thousands of years. The oldest recorded reference indicates that alfalfa was used as forage over 3300 years ago in Turkey. From Iran it came to Greece (Hanson, 1972) and since then it has been an important forage crop for centuries. It was introduced to India about 100 years ago. Here, it is cultivated in Deccan Plateau, humid belt of peninsular India and subtropical regions. It occupies the third largest area of cultivation under fodder crops in India after sorghum and berseem.

Lucerne is one of the most productive and high quality forage legume. Majority of alfalfa grown worldwide is used to feed livestock and is either grazed or harvested and preserved as hay or silage and may also be dehydrated to produce protein supplement to be fed to the animals. It is highly palatable and nutritious to the livestock of all classes. It contains about 15-20% protein, 1.5% Calcium, 0.2% Phosphorous on the dry matter basis and serves as a rich source of vitamin A, B and D. Its importance in the cropping system is many fold as a nitrogen source for the other rotational crops, as a soil improving crop, as a complete source of nutrients for production of meat and milk and as a high quality feed for horses. It has also been recognized for its medicinal value to the sick animals.

A number of adaptive features in alfalfa are particularly favorable for the survival of pasture legumes under herbivore grazing. These include a prostrate growth habit with multiple growing points close to the ground, vigorous regeneration and persistance and a rooting system that can resist being pulled from the soil during grazing. Other desirable features include high palatability, occurrence of tannins in foliage allowing only restricted grazing that assists in preventing bloat in grazing ruminants (Jones and Lyttleton, 1971). Features desirable for efficient commercial seed production in alfalfa include an intensive flowering season, high fertility, self pollination and also the ability to be effectively pollinated by the common regional insect vectors allowing cross pollination and pods which are not spontaneously dehiscent before harvest. The recent breeding efforts have been focussed on forage

quality in conjunction with the release of multi-leaf trait and grazing tolerance (Juan *et al*, 1993 a,b).

Alfalfa is a very complex species from the crop improvement perspective. Complexities of alfalfa breeding include autotetraploid genetics, a myriad of insect and disease problems, perennial growth habit, the necessity of insect pollination, seed production outside the area of forage production and its complexities of nitrogen fixation involving the symbiotic relationship with *Rhizobium*.

Alfalfa has some inherent problems for its genetic improvement, such as the necessity of flower tripping by the insects for pollination, uncontrolled pollination, inbreeding depression, lack of seed production, possibility of hybridization among lucerne plants only at the same level of ploidy. Because of all these hurdles, plant breeders have faced a lot of difficulties in the genetic improvement of this crop through conventional plant breeding methods.

Modern biotechnology methods namely somaclonal variation, anther and pollen culture, somatic hybridization, transformation, etc. are becoming gradually very important as an aid to help the plant breeders in the genetic improvement of alfalfa. In addition to this, several useful genes, e.g., the genes for sulphur containing amino acids in the rumen protein, genes for insect resistance and weed control etc. are being introduced in alfalfa by genetic transformation technique. Standardization of tissue culture and regeneration protocols is an essential component for the application of these biotechnologies.

Medicago species have been found amenable for *in vitro* embryogenesis and plant regeneration. Nevertheless, since each plant is genetically different, much genotypic variation exists in response to *in vitro* manipulation. Identification of regenerating genotypes could be done using optimized protocols and such procedures should be considered as important components of any tissue culture programme with this species as the lack of regeneration potential among the target germplasm sources

may be an early limitation to exploit genetic improvement in lucerne. The present technology is not yet adequate for regenerating full range of alfalfa germplasm.

The applications of biotechnology for the improvement of popular Indian alfalfa cultivars has not been attempted so far and also that alfalfa improvement in India had been attempted marginally. Thus there is a need for identifying genotypes amenable for *in vitro* manipulations among the existing varieties so that they could be improved for fodder yield and quality traits and specific allien genes could be incorporated in their genome. With this background, this study was undertaken with the following objectives.

1. To optimize *in vitro* callus induction and regeneration protocol in different genotypes of alfalfa.
2. To identify genotypes with higher *in vitro* regenerability and
3. To characterize the somaclonal variation.

REVIEW OF LITERATURE

2. *REVIEW OF LITERATURE*

2.1. Tissue culture

The basic foundation of plant tissue culture was laid down with the concept of occurrence of totipotency in plants cells as postulated by Haberlandt (1902) and the success in plant tissue culture was established in 1934 with the pioneering efforts of White and Gautheret independently. A rapid progress was made since then in this area and the techniques of plant organ, tissue and cell culture in a large number of taxa were well established throughout the world and are now being used in numerous areas of plant science.

These techniques have also been used in alfalfa with some degree of success. (Hernandez-Fernandez and Christie, 1989). Production of somatic embryos was first accomplished with the callus derived from one plant from the cultivar Saranac (Saunders and Bingham, 1972). Since then, regeneration of somatic embryos in alfalfa has been achieved from cells in suspension cultures (McCoy and Bingham, 1977), long term callus and suspension cultures (Stavarek *et al.*, 1980) and protoplasts (Dos Santos *et al.*, 1980; Kao and Michayluk, 1980; Johnson *et al.*, 1981 and Mezentsev, 1981). Several authors (Meijer and Brown, 1985 and Chen and Marowitch, 1987) have reported that only a few genotypes in certain cultivars have been found to possess the regeneration capacity. Mroginski and Kartha (1984) emphasised the importance of achieving high regeneration frequency both from primary callus as well as from the cells and calli that have been maintained in cultures for a longer period of time from various experimental materials.

The benefits of incorporating tissue culture techniques into breeding programmes have been discussed by several authors (Scrowcroft and Larkin, 1982; Tomes and Swanson, 1982 and Hammatt *et al.*, 1986). However, number of factors such as, genotype, explant, nutrient media and adjuvants, growth regulators etc. affect callus induction and plant regeneration.

2.1.1. Genotype differences

Tissue culture systems primarily require the germplasm capable of regenerating entire plants from callus and cell suspensions. The regenerated plants must also establish easily in the greenhouse and be able to set seeds. Deineko *et al.* (1992) followed two established protocols for culturing17 *Medicago varia* and 3 *Medicago falcata* varieties on MS media. Three groups of varieties were found with the capacity for regeneration *via* somatic embryogenesis, shoot bud organogenesis and both types of responses.

The genotype specificity of the *in vitro* response of *M. sativa* has been described by several authors. (Mitten *et al.*, 1984; Brown and Atanassov, 1985 and Meijer and Brown, 1985). Somatic embryogenesis in alfalfa was first reported by Saunders and Bingham (1972). This and subsequent reports on plant regeneration from *in vitro* culture have shown that not all alfalfa germplasm have the ability to produce the somatic embryos and plantlets. It is well documented that alfalfa exhibit both intervarietal (Bingham *et al.*, 1975; Atanassov and Brown, 1984; Mitten *et al.*, 1984 and Brown and Atanassov, 1985) and intravarietal (Walker *et al.*, 1978 and Kao and Michayluk, 1981) variation in *in vitro* culture. The embryogenic response was observed as highly genotype dependent and the lack of regeneration potential in most of the germplasm sources has been a major limitation in the application of biotechnology techniques for the genetic improvement of alfalfa (Chen *et al.*, 1987).

The frequency of induction of somatic embryogenesis varied among various cultivars (Bingham *et al.*, 1975 and Brown and Atanassov, 1985). This variation was observed even among the genotypes of a single cultivar (Kao and Michayluk, 1981; Phillips, 1983 and Mitten *et al.*, 1984). Even though the frequency of regenerating genotypes within a cultivar was high, much variation existed in the efficiency of regeneration among them (Mitten *et al.*, 1984). This was attributed to the intervarietal and intravarietal heterogeneity in alfalfa. Fuentes *et al.* (1993) evaluated *in vitro* embryogenic response in 9 lucerne varieties grown in Mexico. All

Cotyledons provided suitable explant source for screening for highly embryogenic genotypes. The correlation between the number of somatic embryos formed by a genotype and the per cent capable of regenerating into plants was low. By screening a wide spectrum of alfalfa germplasm, Brown and Atanassov (1985) and Mitten *et al.* (1984) were able to relate the frequency of *in vitro* plant regeneration to the different germplasm sources.

Somatic embryogenesis exhibited a strong genotype x medium interaction within each cultivar where the total number of embryos varied among genotypes and among madia protocols. The percentage of embryogenic genotypes also varied with the media (Chen *et al.*, 1987). Suginobu *et al.* (1991) concluded that genotype background was a more critical factor for formation of somatic embryos then either the medium or the nature of explant.

In general, tetraploid genotypes of *M.sativa* were generally more embryogenic than plants belonging to the diploid accessions (Meijer and Brown, 1987). The best results from the initial experiments were obtained with hypocotyl and petiole explants showing 70 per cent of the diploid and 94 per cent of the tetraploid genotypes producing an embryogenic response. Varga and Badea (1992) suggested that the application of tissue culture techniques for alfalfa improvements requires screening within specific cultivars in order to identify genotypes capable of regenerating entire plants.

2.1.2. Explants differences

Saunders and Bingham (1972) for the first time regenerated plants in alfalfa from the callus tissues initiated from hypocotyl, internode explants, and immature ovaries through both organogenesis and embryogenesis following a two-step method. Since then callus induction and regeneration of plants in this species through various plant organs and tissues has been studied extensively. In lucerne, various explants for callus induction and regeneration /somatic embryogenesis from different genotypes have been tried by different workers. Many of them have reported the superiority of petiole and hypocotyl explants over the others in respect to callus inductuion and

regenerability (Piccioni *et al.*, 1996). In general, fast growing, meristematic tissues derived from embryos and seedlings were more responsive in culture than those from mature plants (Phillips and Collins, 1979; Novak and Konecna, 1982; Lu *et al.*, 1982 a&b and Ahuja *et al.*, 1983a). Lisunam and Heszky (1987) also reported higher frequency of embryoid formation and regenerative ability in callus or cell cultures derived from petioles than that from culture derived from hypocotyl, cotyledon, stems and leaves.

Young petioles, excised from fast growing young shoots of greenhouse raised mother plants have been the type of explant most often used for establishing embryogenic callus cultures (Xu *et al.*, 1990; Fujii *et al.*1992; Lai *et al.*, 1992; Yu and Pauls, 1993 and Lai and McKersie, 1994). Difference in embryogenic ability might arise if petioles are excised from different parts of the shoot or in different seasons or vegetative stages or it tissues other than petioles are used (Williams and Maheswaran, 1986). This is probably related to the different embryogenic competence of plant tissues at different physiological states, that is, with different abilities to express inherent totipotency or morphogenic capacity (Halperin, 1967). Farago *et al.* (1996) found that recurrent cycles of somatic embryogenesis were induced in calluses derived from petiole segments of 21-day-old seedlings of Slovak lucerne (*M. sativa*) cultivar Lucia.

Mariotti *et al.* (1984) found that root and hypocotyl explants were better than those from the leaves or cotyledon for producing embryogenic callus. Nam and Heszky (1987) found that callus from hypocotyl generally had a higher regenerative ability than that from the other explants. Scarpa *et al.* (1991) reported that hypocotyl provides the best explants for callus production. Lupotto (1983) induced cyclic production of embryoids through secondary embryogenesis from cells of the hypocotyl region of embryoids which were derived from hypocotyl callus.

The high probability (85-100 %) of predicting embryogenic genotypes based upon cotyledon callus response suggested that cotyledons were a good explant source to screen for embryogenic genotypes (Chen *et al.*, 1987). According to Hammad *et al.*

(1993) explants of leaves always gave the best callus and subsequent embryos. However, Okumura *et al.* (1993) reported that shoot tip explants had better ability to undergo somatic embryogenesis as compared to the six other explants tested. Shoot tips had the highest ability to form somatic embryos followed by hypocotyl explants and the frequency of somatic embryo production from cotyledon explants was low.

A stringent correlation had been established between the stage of development of the initial explant, the concentration of 2,4-D and the process of dedifferentiation and differentiation *in vitro* (Denchev and Atanassov, 1988). Interaction between explant and media or between explant and cultivars were significant. The frequency of formation of somatic embryos from the calli induced from the shoot apex was much higher than the frequency of those induced from the petiole, cotyledon, hypocotyl or root tip in one selfed line of embryos but not in the other. This suggested that genotype background was a more critical factor for formation of somatic embryos than either the medium or the nature of explant. Scarpa *et al.* (1993) found that in *M. polymorpha* the morphogenetic response was affected by explant source. Hypocotyl derived callus were the best regenerating tissues. However, the competence of the different tissues in the regenerative pathway has not been completely understood (Finstad *et al.*, 1993).

2.1.3. Hormones, media and adjuvants

It is well known that the concentration and combination of growth regulators govern plant regeneration. The growth regulators, in particular and reduced nitrogen sources exert significant effects on both caulogenesis and *in vitro* regeneration. For most the legumes and some non-legumes, the early stages of somatic embryogenesis have been induced by exposure of tissues to 2,4-D with or without other accompanying growth regulators (Ammirato, 1983). The induction periods vary with the concentration of 2,4-D and with the plant species. Induction periods have ranged from 6 months for the Mitchell cultivar of soybean (Christianson *et al.*, 1983) to as little as 3 to 4 days for alfalfa (Walker and Sato, 1981 and Brown and

Atanassov, 1985). Several other reported methods recommended 2,4-D induction periods between these limits. In many legumes, the removal of 2,4-D and exposure to media lacking hormones or with various combinations of auxin and cytokinin also led to maturation of embryoids and shoot formation. The inductive role of 2,4-D in regeneration of buds and somatic embryo formation has been demonstrated by several investigators (Saunders and Bingham, 1975; Walker, *et al.*, 1979 and Walker and Sato, 1981). However, the induction phenomenon was not specific only to 2,4-D (Kao and Michayluk, 1981) as NAA has been observed replacing 2,4-D in several cases. Stavarek *et al.* (1980) suggested that a medium with a high cytokinin and low auxin content was required to initiate regeneration from long established lucerne callus. Calli induced on kinetin containing media regenerated more than those induced on a kinetin free medium. However, little quantitative information about the *in vitro* response is available (Wang *et al.*, 1988).

Mezentsev (1980) found that tissue culture of excised leaves on modified Gamborg B5 medium resulted in the production of callus from which shoot buds, roots, embryoids and regenerated plants were obtained. Kraic *et al.* (1994) also induced callus from leaf, petiole and stem segments of lucerne on a B5 based medium supplemented with 2,4-D, kinetin and NAA. Morphogenesis in calli was induced on B5 medium containing benzyl adenine phosphate with subsequent placement on a hydrate with growth regulators which ultimately led to formation of shoots. Rooting was induced on rooting medium. Musiyaka *et al.* (1998) observed that tissues of lucerne cultured on B5 medium with 2,4-D, kinetin and NAA initially lost its morphogenetic potential. However, morphogenesis was restored in the calli when cultured on a medium with BAP. An effective method of regenerating plants was the initiation of suspension cultures from callus and the stimulation of embryoid formation by adding amino acids (especially L-argenine) and the yeast extract to the medium. Saunders and Bingham (1972) observed that the callus on its transfer to a medium containing inositol and yeast extract, differentiated into buds profusely and most of which grew into plants.

Mariotti *et al.* (1984) found that of 4 tissue culture media tested, and MSN1 (MS medium+ NAA and BA) UM1 (UM+ 2,4-D and kinetin) were most suitable for callus induction and growth, respectively. Won *et al.* (1999) found that somatic embryos were formed from callus obtained from hypocotyl explants of *M. sativa cv. Vernal* on MS medium containing 4 mg/l 2,4-D + 0.1 mg/l kinetin, or 4 mg/l 2,4-D + 0.5 mg/l kinetin. Mature somatic embryos developed into plantlets after six weeks of culture when subcultured on MS basal medium. Wenzel and Brown (1998) obtained up to 55000 somatic embryos per gram of tissue within 30 days by culturing petioles of a clone of the *M. sativa* variety Rangelander. The petiole explants were exposed for 10 days to 22.6μM 2,4-D and 4.7μM kinetin on MS medium, followed by culture on the same medium containing no growth regulators for 20 days.

Nolan *et al.* (1989) reported that induction of embryo formation occurred on a medium containing 10μM NAA and 10μM BAP and embryo maturation was promoted after transfer to a medium containing 1μM NAA and 10 μM BAP. Shoot development, secondary somatic embryogenesis and occasional plantlet development occurred on a subsequent transfer to 0.1 μM NAA and 1μ M BAP.

Systematic manipulations of the culture protocol leading to somatic embryogenesis in petiole-derived callus in lucerne were performed by Finstad, *et al.* (1993). They demonstrated a requirement for the acquisition of competence prior to the induction of the embryogenesis pathway. Different degrees of competence were apparently acquired by exposure to 2,4-D and NAA. Zhang *et al.* (1995) cultured callus or explants of *M.sativa cv. Jining* in liquid MS medium containing 4 mg 2,4-D and 1 mg BA/litre. Leaves and buds were induced after subculturing, these resistant calli on MS medium with 2 g yeast/litre for 21 days.

Fast embryogenic induction was correlated with high IAA and low ABA levels in the initial explants by Ivanova *et al.* (1994) and it was suggested that a certain balance of growth regulators was necessary to allow the expression of the

embryogenic potential. The consistent stages of the direct somatic embryogenesis were also characterized by changes in growth regulator levels.

According to Romagnoli *et al.* (1996) the best media for somatic embryo production was MS medium + 10 μM 2,4-D + 4.6 μM kinetin. After the auxin shock, the calli were transferred to MS medium containing 10-20 mM NH_4^+ and 30 mM proline. More than 500 somatic embryos per plate were produced. Embryos were grown to plants on MS or half strength MS media.

Cotyledonary abnormalities were observed by Won *et al.* (1999) in secondary somatic alfalfa embryos which were developed from calli cultured on MS medium with various concentrations of 2,4-D. The frequency of normal embryo formation with two cotyledons were above 57 per cent in hormones free or with 0.1 mg/l 2,4-D supplemented which reduced to just 10 per cent with 4mg/l 2,4-D containing medium.

The conditions for callus formation and plant regeneration were confirmed in four cultivars of alfalfa by Kim *et al.* (1999). Among SH (Schenk and Hildebrandt), MS (Murashige and Skoog) and N6 medium (Chu), the SH medium gave the highest efficiencies in callus formation and plant regeneration when supplemented with 3 mg 2,4-D/litre and 5 mg NAA + 2 mg kinetin per litre respectively.

Fujii *et.al.* (1990) produced calli from leaf petioles of *M.sativa* were induced to form embryos on SH medium containing 50 μM 2,4-D and 5 μM kinetin. Calli were transferred to regeneration SH medium containing 10 mM ammonium nitrate and 30 mM proline which induced numerous somatic embryo formation after 3 weeks.

Takamizo *et al.* (1991) developed a simple culture protocol to screen for genotypes with regenerating ability in a recalcitrant Japanese cultivar, Tachiwakaba of lucerne for improving media protocol. Four media (B2, B5h, UM, SH) were compared for ability to induce somatic embryogenesis. Somatic embryogenesis occurred only in

the UM media. The effect of 4 cytokinin (Kinetin, BA, 2ip, Zeatin) were compared in a UM medium supplemented with 2mg 2,4-D/l. BA was more effective than any other cytokinin in term of both callus growth and somatic embryogenesis. The number of somatic embryos formed was greatest at 4mg 2,4-D/l. Somatic embryogenesis did not occur below 2mg 2,4-D/l. The number of somatic embryos formed at 4mg 2,4-D and 0.125mg BA/l was four times as higher as that at 2mg 2,4-D and 0.25mg/l kinetin, as compared to the original composition of the UM medium. They concluded that the UM medium containing 4mg 2,4-D/l supplemented with BA rather than kinetin as a cytokinin was most suitable for somatic embryogenesis in hypocotyl derived calli among all the media, tested.

Parrott and Bailey (1993) initiated callus culture of 300 genotypes of *M.sativa* from leaf, petiole and internodes explants on Blaydes medium containing 10.74 μM NAA, 11.42 μM IAA and 9.29 μM kinetin. Five genotypes produced somatic embryos. Upon transfer of these embryos to the growth regulator free MS medium supplemented with B5 vitamins, new somatic embryos repeatedly formed directly on older somatic embryos without an intervening callus phase in a cycle lasting about 30 days. Glucose, Maltose and fructose stimulated recurrent embryogenesis more effectively than sucrose.

Walker and Sato (1981) found that adequate levels of reduced nitrogen in the form of ammonium ions were required for embryogenesis after its induction with 2,4-D. As reported in carrot by Ammirato (1983), somatic embryogenesis in morphogenetically competent cell lines of alfalfa also could be inhibited by withdrawal of reduced nitrogen after the initial induction. A minimum concentration of 12.5mM ammonium was optimum for embryogenesis and at 50mM ammonium, embryoids were produced even in cultures exposed to a rhizogenic hormone combination in the presence of nitrate. Stuart and Strickland (1984 a, b) studied the role of amino acids in somatic embryogenesis in *M. sativa*, and found that the addition of L-proline to SH medium, (which already contained ammonium) increased embryoid formation. In most other reported cases of embryogenesis in alfalfa, further development of embryoids occurred in media

supplemented with either yeast extract or in MS based media containing high levels of nitrogen.

Walker and Sato (1981) suggested that exogenously supplied ammonium ions were critical for *in vitro* morphogenesis. Optimum embryoid formation in the lucerne variety Regen S required a minimum of 12.5mM ammonium ions in the regeneration medium, while root formation occurred in the absence of such ions and was inhibited by ion concentration of 50mM and above.

Stuart and Strickland (1984) also observed that the proline-enhanced regeneration of somatic embryos was found to depend upon the ammonium ion. Optimum regeneration occurred when 10mM proline and 25mM ammonium was added to the embryogenesis medium. Glutamine was only the amino acid capable of stimulating somatic embryogenesis which was not dependent on ammonium and it could be substituted for ammonium in proline stimulated embryogenesis.

Meijer and Brown (1987) observed that ammonium was essential for embryoid induction and differentiation with 5mM being the optimum for induction and 10-20mM optimum for differentiation. Exogenous amino acids were not essential for differentiation and were often inhibitory, except for 1 or 2g CH/l or 4.4 mM glutamine with 3.1mM proline which, under certain conditions, resulted in the increase of 20-30 per cent in the number of embryoids obtained. High and low sucrose concentration inhibited somatic embryogenesis.

In a study by Shetty and McKersie (1993), somatic embryogenesis was induced in petiole derived culture in the presence of 1mg 2,4-D and 0.2 mg/l kinetin and embryo elongation occurred when embryogenic calli were transferred to hormone free medium. The addition of 10-25 mM proline and 0.1-0.5 mM thioproline, sulphur containing proline analogue to the induction medium stimulated 2,4-D induced somatic embryogenesis. At higher concentration (0.5-1.0 mM) thioproline decreased the size of embryogenic callus, reduced embryo number but increased the size of somatic embryos. Potassium stimulated the size of embryogenic callus and

promoted embryo formation when its level was increased from 25 mM to 75-100mM in the induction medium. Addition of 10-20 mM proline with 0.4 mM thioproline and 50 mM potassium enhanced the size of embryogenic callus and significantly enhanced subsequent embryo formation along with higher number of cotyledonary embryos.

Stuart *et al.* (1984) found that addition of L-proline to a medium developed by Saunders and Bingham (1972) led to a three fold increase in embryoids formation. Proline acted synergistically with NH_4^+ and embryogenesis was highest with 100 µM proline and 25 µM NH_4^+. When alanine, glutamine, arginine and asparagine were substituted for proline, the increase in the regeneration was less, but the embryos were of a better quality as measured by size and frequency of regeneration to plantlet. Mezentsev (1981) found improvement in embryoid formation by adding L-arginine (1.7g/l) to the medium.

Lupotto (1983) showed that although the initiation of somatic embryogenesis started on simple hormone-free medium, a more complex medium with yeast-extract was needed to propagate embryogenic cultures.

Strickland *et al.* (1987) recorded that maltose, maltotriose and soluble starch increased somatic embryo yield and plantlet regeneration frequency from petiole derived callus. The effect of maltose proved dependent on the presence of $NH4^+$. A maltose based medium containing $NH4^+$ and amino acids, especially proline, proved effective for regeneration and a clone S1-9, with low regenerative ability, also responded to maltose by increasing somatic embryo production.

Nichol *et al.* (1991) pretreated the callus cultures initiated from petioler explants of clone RA3 of *cv.* Regen-S with various organic acids for one subculture cycle prior to somatic embryo induction with 2,4-D and regeneration on hormone free medium. This resulted increase in embryo yield after induction and regeneration of the callus. Sreedhar and Bewley (1998) indicated that nitrogen and sulfur supplementation in the callus induction medium had a positive effect on the amount

and nature of callus produced per explant. The improved somatic embryos accumulated starch equivalent to about 6 per cent and the lipid equivalent to about 17-20 per cent of the embryo dry weight.

2.1.4. Regeneration

Somatic embryogenesis in *M.sativa* was first reported by Saunders and Bingham (1972). Since then several reports have been published on the regeneration of this species from callus, cell suspension and protoplast cultures (McCoy and Walker, 1984). Some clones showed the ability of forming somatic embryos directly from mesophyll protoplasts (Kao and Michayluk 1980 and Dijak and Brown 1987). Somatic embryogenesis could occur in tissue or cell culture of a number of monocotyledonous and dicotyledonous species, usually in response to the presence of exogenous plant growth regulators, although other medium components and explant or cell types were also important factors (Ammirato 1983). This *in vitro* morphogenic pathway involved the formation of bipolar structures, resembling zygotic embryos, with both a shoot and a root meristem as opposed to organogenesis in which shoot (or root) formation was induced. Initially, somatic embryos were thought to arise exclusively from single cells (Haccius, 1978), but some publications have casted doubt on the validity of this hypothesis (Wernicke *et al.*, 1982 and dos Santos *et al.*, 1983).

Callus, cell suspension and protoplast derived cultures of *M.sativa* regenerated *via* somatic embryogenesis (Saunders and Bingham, 1972; Bingham *et al.*, 1975; Dos Santos *et al.*, 1980; Johnson *et al.*, 1981; Kao and Michayluk, 1981; Novak and Konecna, 1982; Xu, *et al.*, 1982 and Atanassov and Brown, 1984). The process was truly embryogenic. Histological examination of embryogenic tissues showed that the embryos arose from single cells of the parental explant (dos Santos *et al.*, 1983). Long-term embryogenic cultures of lucerne could be initiated on hormone–free medium and propagated on medium containing yeast extract (Lupotto, 1983). Saunders and Bingham (1975) studied the influence of growth regulators on bud regeneration. A common feature of the successful methods for plant regeneration in

alfalfa was the use of 2,4-D for explant dedifferentiation while embryo formation was stimulated by various organic (Stuart and Strickland, 1984 a&b) and also inorganic (Walker and Sato, 1981) compounds.

Walker *et al.* (1978) obtained regeneration through shoot bud organogenesis in *M. sativa* L. *cv. 'Regen'* by transferring callus from induction medium containing growth regulators to a regeneration medium lacking of growth regulators. They suggested that the determination of organ type occurred principally on regeneration medium. These observations were consistent with the hypothesis advanced by Bonnett and Torrey (1965) for *Convolvulus* which stated that organ induction and organ determination might occur sequentially and required different conditions for each. During organogenesis in alfalfa the process of organ induction might initiate on induction medium and the determination of organ type could take place on regeneration medium. Churova (1981) observed that growth centers were induced and stalk meristems differentiated and developed into rootless plants (shoot stocks) in MS medium. He also successfully attempted to induce shoot bud organogenesis in callus cultures of *M. sativa* in Blaydes medium. Walker *et al.* (1979) observed the temporal separation of induction and differentiation phases of organogenesis and identification by size of classes of cell aggregates which were morphogenetically capable of inductive responses for organogenesis.

Dos Santos *et al.* (1983) suggested that embryoids originated from groups of apparently homogeneous meristematic cells on organized callus, while on friable callus they originated form single embryogenic cells distinguishable from the non embryogenic ones by their staining reactions. They provided evidence for the origin of embryoids from single cells in the epidermis of cotyledons, hypocotyls and roots of induced plantlets as well as in friable callus of *M. sativa*. Bingham and McCoy's (1986) observation that regeneration in alfalfa occurred *via* organogenesis and embryogenesis, was based on the morphology of regenerating structures.

Gazaryan *et al.* (1993) found that regenerants were produced on B5 agar medium and their peroxidase (Px) activity was measured. Embryogenic cell cultures had

higher Px activity than those in which the regeneration process stopped, although cell division and growth continued. Isoelectric focusing of Px showed a predominance of acidic Px isoenzymes (and only traces of alkaline ones) in embryogenic callus in contrast with the presence of both acidic and alkaline isoenzymes in non-embryogenic callus.

Liu *et al.* (1993) produced adventitious roots and buds on differentiation media from the cotyledon and hypocotyl derived calli of *M.sativa* extracted total soluble proteins at various stages of organogenesis and embryogenesis and separated by SDS-PAGE. Proteins with MW of 110 and 80 kD were related to bud and root differentiation,respectively.

Musiyaka *et al.* (1998) cultured the tissues of a lucerne cultivar on Gamborg B5 medium with 3 per cent sucrose and 4mg 2,4-D, 4mg kinetin and 0.25mg NAA/l completely lost its morphogenetic potential after 12-14 subcultures. Culturing the callus on B5 medium with 0.5mg 2,4-D and 50mg NAA/l restored morphogenetic potential. Buds and roots formed after 30 days culture on a medium inducing morphogenesis (with 0.2mg BAP/l). Nikolic *et al.* (1985) observed that callus induction, bud formation, shoot elongation and rooting in lucerne were obtained on sequence of media with different hormone additions. Regeneration capacity declined after several months in cultures.

Iantcheva *et al.* (1999) developed of a simple and rapid procedure for direct somatic embryogenesis from wild *Medicago* sp. (*M. truncatula, M. littoralis, M. murex and M. polymorpha*) and exploited various explants including those of meristematic zones. Phytogel solidified medium supplemented with thidiazuron or 6-BAP at different concentrations effectively promoted this process. Histological analysis confirmed the nature of the directly formed somatic embryos. Secondary embryogenesis was also observed. Regenerated plants were easily developed with well-developed root systems on medium with reduced levels of macro elements and sucrose.

Shao *et al.* (2000) developed two simple, rapid and efficient protocols for the regeneration of transformed tetraploid lines of alfalfa. In the first regeneration system (the MSH system), inoculated leaf explants were incubated on MS medium supplemented with 2,4-D and kinetin and then subcultured onto plant growth regulator-free MS medium to induce direct somatic embryogenesis. In the second regeneration system (the B5h system), the inoculated explants were incubated on B5 medium with hormones to induce indirect production of somatic embryos *via* embryogenic callus. In both systems, an effective kanamycin selection regime was employed and was maintained when the embryos were subcultured onto a recovery medium (Boi2Y) to promote further embryo development. The use of Boi2Y medium was particularly important for shortening the regeneration time and promoting a higher frequency of healthy plantlet production from the somatic embryos.

Three factors to control alfalfa regeneration (*via* somatic embryogenesis) *in vitro* have been proposed (Walker *et al.*, 1978; 1979 and Walker and Sato, 1981). They were the structural quality and quantity of the exogenous growth regulator used as auxin source, the concentration of exogenous cytokinin and the level of reduced nitrogen in the regeneration medium. The use of plant regeneration by somatic embryogenesis offered the possibility for reducing the time required to produce new varieties (Novak and Konecna, 1982 and Varga and Badea, 1992) and the introduction of important agronomic traits by employing the current techniques of molecular biology and plant transformation (Hill *et al.*, 1991 and D'Halluin *et al.*, 1990).

Recurrent somatic embryogenesis (RSE) of alfalfa (*Medicago sativa* L.) was originally described by dos Santos *et al.* (1983) and Lupotto (1983,1986). This phenomenon occurred either on Blaydes (Lupotto, 1983) or MS (dos Santos *et al.*, 1983 and Lupotto, 1986) media that were devoid of growth regulators, but supplemented with yeast extract. In Lupotto's system, somatic embryos of alfalfa continuously gave rise to new embryos, without an intervening callus phase, in a 20 days cycle. The somatic embryos would germinate, producing roots and primary

leaves, but would cease growth as soon as somatic embryos appeared in the crown region. Although it was difficult to convert the embryos into plants. However, the plants could be recovered by excising the shoots from germinating embryos and allowing them to root on growth regulator-free medium. Some plants could also be obtained by placing the somatic embryos directly in to soil.

A large number of legumes show genotype-specific regeneration as indicated in studies on alfalfa (Bingham *et al.*, 1975; Phillips, 1983 and Mitten *et al.*, 1984). Bingham *et al.* (1975) embryogenic capacity was controlled by two loci and therefore it was easy to incorporate it into cultivars by means of recurrent selection or by backcross breeding. Increased regeneration was found in the Regen-S cultivar of alfalfa, developed by two cycles of recurrent selection for regeneration capacity. Reisch and Bingham (1980) found that in diploid alfalfa, bud differentiation from callus was controlled by two dominant genes, and both were required to be present in order to obtain more than 75 per cent regeneration. Wan *et al.* (1988) studied 7 tetraploid lucerne cultivars and they also suggested that regenerability in this system was controlled by 2 complementary dominant genes, both were necessary for regeneration. Gene dosage influenced regeneration efficiency and cytoplasmic effects influenced the interaction between callus induction medium and regenerability. Walton and Brown (1988) found evidence of cytoplasmic inheritance for extent of embryogenesis in 2 reciprocal crosses of *M.sativa.*

Hernandez-Fernandez and Christie (1989) examined F_1 and S_1 segregation ratios of three alfalfa genotypes which differed in their ability to initiate callus. They concluded that callus initiation was a single-locus-controlled trait showing complete dominance and random chromatid segregation. Embryogenesis among the plants producing callus was controlled by 2 complementary loci with additivity within each locus. Okumura *et al.* (1994) observed the relationships between regeneration and productivity of somatic embryos with a view to developing plants with high productivity of somatic embryos. Effects of somatic variation were investigated for important traits.

2.1.5. Somaclonal variation

One of the applications of tissue culture is the exploitation of genetic changes occurring in plants regenerated from callus and protoplasts for producing agronomically useful variants (Larkin and Scowcroft, 1981 and Evans and Sharp, 1983). Plants regenerated from tissue culture might quite often vary from the tissue donor plant in one or more characteristics. This variation was termed as somaclonal variation by Larkin and Scowcroft (1981). Though a large amount of natural variability is known to be present in alfalfa, it has been very difficult to improve forage yield in comparison to cereals and food crops (Bingham, 1981). Somaclonal variants for qualitative genetic alternations (Bingham and McCoy, 1986), disease resistance (Latunde-Dada and Lucas, 1983 and Johnson *et al.*, 1984) and quantitative traits such as herbage yield (Johnson *et al.*, 1984 and Pfeiffer and Bingham, 1984) have been reported in alfalfa. Among the quantitative agronomic traits, only forage yield has been taken into account. An indication of the possible utilization of somaclonal variation in the genetic improvement of alfalfa could be derived from comparison of ranges of variations observed in the regenerants and original populations.

Genetic variability for *in vitro* callus proliferation (reported as callus fresh weight, callus growth rate, callus production and callus diameter) has also been found. Brown and Atanassov (1985) evaluated numerous alfalfa cultivars to determine the role of genetic background in the *in vitro* culture of *Medicago*. A wide range of callus yield was observed among cultivars ranging from 62mg for *cv. Chilean* hypocotyl explants to 720mg for *cv. Angus* cotyledons. Wide variation for callus growth among genotypes within some cultivars were also detected.

Genetic variation often arose in genetically closed systems in which novel genes have not been introduced from outside sources. This could be exemplified by the frequent recovery of somaclonal variants among plants regenerated from tissue culture (Larkin and Scowcroft, 1981 and Ogihara, 1981). In alfalfa, somaclonal variation occurred as a common phenomenon and an array of variation involving flower colour, leaf morphology, branching patterns, fertility, regeneration ability,

vigor and ploidy level were observed among regenerated plants (Reisch and Bingham, 1981; Bingham and McCoy, 1986 and Groose and Bingham, 1986).

Although much effort has been directed towards generating somaclonal variants, little has been known about the genetic mechanisms that give rise to this variation. Several types of genetic changes have been proposed to account for the somaclonal variation, including changes in chromosome number, translocations, chromosome reduction or duplication, somatic recombination, movement of transposable elements, duplications or deletions of chromosomal segments and point mutations (Larkin *et al.*, 1989). Some of these changes involved chromosomal aberrations that were cytologically detectable and changes in ploidy level, aneuploidy and translocations have been observed in many regenerated plants including alfalfa (Johnson *et al.*, 1984). Reports on cytological analysis of genome rearrangements in alfalfa, however, have been limited due to the size of chromosomes and the extent the structural changes. Hence, more sensitive methods are required to detect subtle rearrangements of DNA in regenerated alfalfa plants.

The frequency of certain chromosomal variants differed with the ploidy level of the donor. When diploid alfalfa was regenerated, the most common type of chromosome variation was spontaneous doubling. When tetraploids were regenerated from callus cells or protoplasts, both aneuploidy and chromosome doubling were common and some chromosome structural changes occurred.

Initially, gross karyotypic changes such as aneuploidy were assumed to be responsible for all variations observed among regenerated plants (Murashige, 1974). More subtle alterations such as chromosome breakage and reunion, translocations, deletions, duplications and transpositions were subsequently identified (Larkin *et al.*, 1989) and variations were observed among regenerants with euploid chromosome number. Several mitotic mechanisms have been proposed to account for subtle genetic rearrangements in somaclonal variants. Asymmetrical mitotic exchanges which could be promoted by the presences of long tandem repeated sequences, could result in somatic segregation for duplications and deletions of

repeated sequences included in the exchanged segments. Loss of homologs owing to somatic reduction or duplication also might account for changes in copy number of repeated DNAs. Breakage events have been implicated as a primary source of chromosome rearrangements in regenerated plants (McCoy *et al.*, 1982 and Brettell *et al.*, 1986), and breakpoints were frequently located on chromosome arms containing large blocks of heterochromatin (Lee and Phillips, 1987). Heterochromatic regions are frequently composed of non-transcribed, repeated DNA sequences (Appels *et al.*, 1978; Peacock *et al.*, 1981 and Johnson *et al.*, 1987). Lee and Phillips (1988) have suggested that late replication of these regions during mitosis might be the cause of chromosome breakage during *in vitro* culture. Most chromosome breakage events result in chromatin loss; however, a cell might incorporate a chromosome fragment into its genome resulting in chromatin gain.

Mechanisms of somaclonal variation identified in alfalfa include changes in chromosome number and structure, dominant and recessive mutations, transposable elements and changes in chloroplasts and mitochondria. Importantly, a given variant may carry more than one nuclear or cytoplasmic mutation.

Major quality considerations of alfalfa improvement include quality and quantity of proteins and the need to reduce the components that cause bloat. Alfalfa protein is of fairly good quality although noticeably deficient in methionine. Cell culture technology has recently been utilized in an attempt to isolate amino acid over producers (Maliga, 1980). Selection for resistance to ethionine (an analog of methionine) resulted in a cell line with a tenfold increase in soluble methionine, a 40 per cent increase in free methionine and protein amino acids (Reisch *et al.*, 1981). Ethionine resistance was maintained in some plants following plant regeneration, though its inheritance has not been determined.

A field study of herbage yield of 32 diploid and 16 tetraploid regenerates of HG2 alfalfa revealed variants for herbage yield (Reisch and Bingham, 1981). Herbage yield is considered a quantitative genetic trait and is controlled by many genes, each of which having small effect on yield. It is also well known that single qualitative

genes such as those for dwarfism could override the quantitative system. Variants in herbage yield due to both quantitative and qualitative changes in HG2 were identified in the field study. The most outstanding variants for herbage yield (NS1) was regenerated from an unmutagenized cell line. Variant NS1 yielded threefold more dry matter in a clonal evaluation of spaced plants than 4x HG2. This was a significant increase in herbage yield. Clone NS1 was a tetraploid and its superiority over 4x HG2 was confirmed in a follow-up study to compare the best somaclonal derivative of HG2 with the best sexual derivatives of HG2 (Pfeiffer and Bingham, 1984).

Somaclonal variation has several unique applications in alfalfa. These include production of chromosomal and genetic variants in a common genetic background, potentially enhanced gene transfer in species hybrids *via* chromosomal interchanges or DNA transposition, and a novel method of mapping genes to chromosomes.

Somaclonal variants of alfalfa were noted among the first alfalfa plants regenerated from callus (Saunders and Bingham, 1972). Johnson, *et al.* (1980) reported that thirty-two regenerated plantlets were phenotypically indistinguishable from the plant of the lucrene variety Regen S1 from which they were regenerated. However the SI seedlings progenies of selected regenerated plants were significantly more stunted and were more variable in cotyledon number and shape, and leaf morphology than those of the original plants.

Groose and Bingham (1984) obtained plants from hypocotyl derived callus of two tetraploid alfalfa genotypes bred to be heterozygous for four heritable traits and 21 per cent of the regenerants were variants for one or more characters. They reported heterozygosity for several traits and at least 11 per cent of the regenerated plants lost one or more chromosome in the first cycle. Approximately 60 per cent of variants regenerated plants exhibited a change in chromosome number. Reculture of 28 chromosome variants did not result in a shift toward euploid among 113 plants regenerated.

When a white flowered alfalfa mutant regenerated from tissue culture of a purple flowered plant was recultured, more than 20 per cent of the regenerated plants had purple flowers. The reversion occurred early in culture and it was suggested that it might have resulted from genome shock associated with callus formation. Reversion also occurred in plants but at a much lower frequency. The mutation was transmitted to a sexual progeny which also reverted *in vitro* (Groose and Bingham, 1986). In an experiment concluded by Nikolic *et al.* (1986), the *in vitro* regenerated plants obtained did not differ from normal plants of the variety in their morphological traits and yield. It was thought that *in vitro* methods could be successfully used in further breeding work of lucerne. Arcioni *et al.* (1988) concluded that somaclonal variation was genotype specific.

Electrophoretic analysis of proteins revealed that 2 out of 22 alfalfa somaclones derived from one parent plant differed significantly from the parent and the other somaclones (Baertlein and McDaniel, 1987). Plantlets derived from 3 calli from a single plant were compared using discriminant analysis based on protein pattern variations. Somaclones could be grouped by lineage with 80 per cent accuracy. Two of the somaclone linkage groups showed no overlap with the parent group.

Hartman, *et al.* (1984) found that somaclonal variation for disease resistance in alfalfa was genetically transmissible and appeared to be due to dominant mutation Latunde-Dada and- Lucas (1988) found that disease severity in the regenerants population was reduced in comparison with parental controls.

Ptackova *et al.* (1988) observed, significant differences in water holding capacity in 40 individuals derived by somatic embryogenesis from a single *M.sativa* plant. Some of them combined drought resistance with high chlorophyll content. Safarnejad *et al.* (1996) observed that one somaclone in particular (6R21V) when compared with the parent line, showed increased salt tolerance, greater accumulation of proline and a greater increase in the antioxidant enzyme, glutathione reductase.

Koike *et al.* (1992) observed that one somaclone had significantly higher dry matter yield than the parent. Variation was also observed in peroxidase and esterase isoenzymes. Varga and Badea (1992) suggested that by using somaclonal variation, it was possible to shorten the time needed for the development of new alfalfa varieties if *in vitro* regenerated variant plants were used as source material.

Deineko, *et al.* (1997) reported very wide variations of morphological characteristics. Qualitative variation in the composition of seed storage protein was lacking, but a significant difference in the frequencies of some individual storage protein was shown. This might be related to some modifications in gene expression induced by plant culture *in vitro*.

The results of Kidwell and Osborn (1993) in alfalfa somaclones indicated specific repeated nuclear DNA sequences and changed copy numbers in plants regenerated from tissue culture. Piccioni *et al.* (1997) quantified the incidence of somaclonal variation estimated by RADP fingerprinting in *in vitro* cultured plantlets of the highly regenerative genotype A70-34 (Synonym RL-34) of *M.sativa* from the cultivar Rangelander. Plantlets obtained by axillary branching propagation on a growth regulator free medium were compared with those obtained through indirect sometic embryogenesis, *i.e.*, from somatic embryos regenerated from callus proliferated from petiole tissue. Plantlets derived from axillary branching exhibited no variants for any of the 75 RADP markers obtained using different light (decamer) primers even after 12 repeated monthly subcultures. In contrast, the RADP fingerprints of 9 out of 39 plantlets regenerated by indirect somatic embryogenesis differed from that of the donor for at least one primer and one polymorphic amplification product. The light primers generated 19 new RADP markers in the somaclonal variants that were not found in the donor plant fingerprints, while 24 RADP markers present in the donor plant fingerprints were not scored in the somaclonal variants.

2.1.6. Biochemical variation in somaclones:

(a) Isozyme variation: Several reports on the use of isozyme analysis for characterization of genotypes, ecotypes and phylogeny of lucerne are available. In some studies isozyme profiles of *in vitro* cultures have also been reported.

Embryogenic cell cultures obtained from primary calli of lucerne had higher peroxidase (Px) activity than those in which the regeneration process had stopped. The regenerants maintained high Px activity in culture conditions. Isoelectric focusing of Px showed that in embryogenic callus had a predominance of acidic Px isoenzymes in contrast with presence of both acidic and alkaline isoenzymes in nonembryogenic calli (Gazaryan *et al.* 1993).

Variation in PAGE banding patterns of peroxidase and esterase isozymes in 10 *M. sativa* genotypes grown under different environmental conditions allowed their clustering into three groups (Zhang and Hao, 1993). The peroxidase (POD) isoenzyme was analysed by PAGE in shoot tips of 11 accessions of *M.sativa* and 11 accessions of *M. ruthenica* (Yang *et al.*, 1994). They found 6 band types of POD isozymes in 11 *M. sativa* accessions. Similarly six enzyme systems were analysed using horizontal starch gel electrophoresis in 11 populations originating from north Tunisia (8 of *M.ciliaris* and 3 of *M. intertexta*). The polymorphism indices and Nei's genetic distance revealed higher levels of genetic variability within the between populations of in *M. intertexta* than *M.ciliaris*. Cluster analysis, based on Nei's genetic distance, revealed a net separation between the 2 taxa. However, one population of *M.intertexta*, originating from Sedjnana, was enzymatically similar to *M.ciliaris*. This result indicated possible introgressive hybridization between *M.intertexta* and *M.ciliaris* (Cherifi, 1996).

Electrophoretic analysis of leucine amino peptidase (LAP), peroxidase and fluorescent esterase was used to estimate allele frequencies and genetic diversity in 23 Chinese and 9 American cultivars of lucerne. All the Chinese cultivars had 4 alleles at the LAP-2 locus. Genetic variation of Chinese cultivars was mainly

interspecific, displaying uniformity among the cultivars and heterozygosity between the cultivars, indicating a relatively narrow genetic background. Chinese cultivars had a lower genetic variation among populations than between populations, suggesting extensive ecological adaptability and diversity (Lu, 1997). Liu *et al.* (1992) analysed esterase and peroxidase isozymes using PAGE and isoelectrofocusing for cultivar identification of alfalfa.

Khavkin (1991) found in *Zea mays* that variable bands of anodic esterase and peroxidase were noted in zymogrames of leaf extracts from second generation (Sc2) maize somaclones. Some of the bands were found active in the somaclones but absent from the samples of the mother plant (standard). Some loci appeared to be more susceptible to somaclonal variation than the others. The data indicated that there might be a specific control system ordering tissue and organ specific isoenzyme spectra which might be affected by somaclonal variation at the early stages of a regulatory cascade affecting cell and tissue differentiation.

Amberger *et al.* (1992) found that the recovery of two isoenzyme variants from progeny of 185 soybean plants regenerated from somatic embryogenesis indicating the feasibility of selection for molecular variants. Henn *et al.* (1993) observed no significant differences in isozyme patterns of *Solanum tuberosum* for peroxidase, acid phosphatase, 6- phosphogluconate dehydrogenase and glutamate oxaloacetate transaminase (aspartate aminotransferase) while slight differences were observed for esterase isoenzyme patterns. Liu *et al.* (1993) found that differences were observed in the peroxidase isoenzyme profiles in both spontaneous and induced somaclonal variants in *Iris*.

Singh *et al.* (1995) observed that in *Cajanus cajan* when the primary callui were cut in to small pieces and subcultured, they displayed variation in isoenzyme patterns after 30 days. Out of the 105 subculture analysed, 17 were variants for either acid phosphatase or esterase, while one subculture was a variant for both. The frequency of variant subcultures differed between genotypes being lowest (14.38%) in ICP6974 and highest (19%) in ICP7182.

The isocitrate dehydrogenase, acid phosphatase, peroxidase and esterase enzyme systems could be considered as good markers for investigating possible genetic variations in plant populations of *Cereus peruvianus* because of their variants which developed in *in vitro* cultures and were carried over to regenerated plants (Mangolin *et al.* 1994).

Binsfeld *et al.* (1996) observed the differences in electrophoretic patterns of peroxidase and esterase enzyme systems among the plants developed *in vitro* and the control (plant) in *Solanum tuberosum*. The isoenzymatic variability found in the somaclones indicated the efficiency of callus culture in the induction of somaclonal variation. The different response among the somaclones from each cultivars were attributed mainly to genotypic differences.

Pupilli *et al.* (1991) confirmed hybrid nature of calli developed after electrofusion of protoplasts of *Medicago arborea* and *M. sativa* by isozyme analysis. Nenz *et al.* (1996) further continued this study and established the hybrid nature of this interspecific somatic hybrid by isozyme analysis.

(b) RAPD variation in somaclones :

Echt, *et al.* (1992) found that RAPD markers appeared useful for the rapid development of genetic information in species like lucerne where little information existed.

Yu and Pauls (1993) summarised that DNA bulking and methods for comparing RAPD patterns would be greatly useful for identifying cultivars for studying phylogenetic relationships and for selecting parents to maximize heterosis in crosses.

Yu and Pauls, (1993) identified random amplified polymorphic DNA (RAPD) markers linked to genes controlling somatic embryogenesis in *M.sativa*.

Segregation analysis of the somatic embryogenesis trait and RAPD markers in an F1 population of 83 plants was carried out. Brummer (1995) studied that at least 10 RAPD primers appear to be necessary in order to develop reliable estimates of relatedness among *Medicago* accessions. Bonnin *et al.* (1996) used RAPD markers and one morphological marker to study the structure of *M. truncatula* among and within population.

Piccioni *et al.* (1997) estimated the incidence of somaclonal variation by RAPD fingerprinting in *in vitro* cultured plantlets of a highly regenerative genotype of *M. sativa.* The RAPD fingerprints of 9 out of 39 plantlets regenerated by indirect somatic embryogenesis differed from that of the donor for at least one primer and one polymorphic amplification product. The eight primers generated 19 new RAPD markers in the somaclonal variants that were not found in the donor plant fingerprints, while 24 RAPD markers present in the donor plant fingerprints were not scored in the somaclonal variants. Most of the somaclonal variants displayed one to five polymorphic bands. Moreover, six of nine somaclonal variants were polymorphic, with respect to the donor plant, with two or more primers. RAPD markers were an efficient tool for the early detection of somaclonal variants in tissue culture.

Phan *et al.* (1996) demonstrated the occurrence of somaclonal variation in the material used to produce protoplasts for the gene transfer in *Oryza sativa* by the occurrence of extensive DNA changes in the cultured cells.

In *Hordeum vulgare,* RFLP and RAPD with twenty decamer primers were used to determine the variability induced by tissue culture. Polymorphism was detected in sequences coding for C-hordeins in a line derived from the cultivar, *Jubiley.* Two lines from cultivar Ruen exhibited polymorphic bands after hybridization with a mitochondrial DNA probe (Todorovska *et al.* 1997).

Nenz *et al.* (1996) obtained somatic hybrid plants by symmetrical electrofusion of protoplasts of *M. sativa* and *M. arborea*. They established the hybrid nature of these plants by RFLP analysis.

2.1.7. Chemical composition/Nutritional composition :

The chemical composition of a feed gives a general idea about its usefulness. The actual nutritive value can be gauged only through animal feeding to find out the extent of nutrients present in the feed are being digested, assimilated and metabolized within the animal body. The digestibility and more particularity the energy and protein value of the herbages are high when the plant is young and they diminish as it matures.

However a compromise has to be effected regarding to the time of harvesting a crop so that more herbage yield or tonnage, though of somewhat diminished digestibility, could be obtained.

The chemical components of ingested forage affect the digestibility and nutritive value of the forage and certain compounds interact with physiological reaction in the animal resulting in toxicity or animal stress. The fibre, particularly with regard to forages, has traditionally been referred to the complex of dietary nutrients which are relatively resistant to digestion and are slowly and only partially degraded by ruminants (Van Soest, 1982 and Cherson and Forsberg, 1988). By the definition cellulose, hemicellulose and lignin are the major components of fibre.

The neutral detergent fibre and acid detergent fibre concentration of forages provide useful information about quality. High quality forage is characterized by relatively low concentration of both NDF and ADF. Effects of NDF as intake are manifested primarily through effects on indigestible NDF.

Gupta and Pradhan (1974) evaluated a number of legume and non-legume forage crops for their relative nutritive value. They found that lucerne had 17.80 % crude

protein, which was more than berseem and cowpea. Cell wall contents (NDF, ADF, cellulose, hemicellulose and lignin contents) were 46.00, 32.00, 21.50, 14.00 and 9.60 %, respectively in the evaluated Lucerne forage.

Tewatia *et al.* (1998) reported that lucerne had 20.30, 43.92, 34.90, 24.41, 9.02 and 9.37 % of crude protein, NDF, ADF, cellulose, hemicellulose and lignin, respectively. In another study these workers found that lucerne hay had 17.10 % CP, 3.40% EE, 2.30% CF and 10.40 % ash contents. This variation in CP values may be attributed to variation in crop maturity stage.

Two varities of lucerne (T-9 and LH-84) were evaluated by Panwer *et al.* (1992) for their nutritive value in Beetal kids. The chemical constituents of both varieties were comparable. The concentration of CP, NDF, ADF, CF and ash was 15.66, 70.05, 53.47, 24.12 and 10.78% for T-9 and 16.17, 67.65, 46.47, 22.18 and 9.60% for LH-84, respectively. Wide variation in the chemical composition of lucerne grown at different places in the country has been given by Sen and Ray (1971). They reported that chemical constituents vary with the different stages of crop harvesting. Maximum crude protein contents upto 26 % have been reported in lucerne grown at Punjab. Similarly the lowest crude protein contents (13.31%) were also reported from the same place. CF contents varied from 18.39-43.66% in the lucerne grown at different regions of the country. These workers reported ash contents as low as 8.83 and as high as 17.66%. However, the information on the nutritive value of somaclones of forage crops evolved through tissue culture seems to be frugal.

MATERIAL AND METHODS

3. *MATERIAL AND METHODS*

The details of material used in the present study and the methods adopted in various experiments are described hereunder.

3.1 Material

The experimental material consisted of the following 8 genotypes of lucerne obtained from Indian Grassland Fodder and Agroforestry Research Institute, Jhansi.

1. C-10
2. IG-1212
3. IL-75
4. A-3
5. LLC-9
6. LLC-3
7. Anand-2
8. AL-95-12

3.2 Sterilization of glasswares and instruments: All the glassware's were cleaned with teepol and washed under running tap water, rinsed with distilled water and then air dried followed by oven drying for 2 hours at 150°C. Scalpels, needles, forceps and other small instruments were thoroughly cleaned with teepol followed by rectified sprit, wrapped in aluminium foil and kept in a clean sterilized box and then sterilization was done in autoclave at 15 psi (121°C) for 20 minutes.

3.3. Explants

The explants used for *in vitro* culture in the present studies consisted of 5-7 days old and hypocotyl, epicotyl and cotyledon derived from *in vitro* germinated seeds.

Sterilization of the explants and inoculation:

Explants were surface sterilized before inoculation onto the medium to kill the microbes adhered to the explants which would otherwise contaminate the medium and suppress the explant response. All the following operations were done in a sterile laminar air flow cabinet to ensure aseptic conditions.

Hypocotyl, epicotyl and Cotyledon explants:

The mature seeds were surface sterilized as described above and germinated on MS basal medium. After the required period (5-7 days), the hypocotyl, epicotyl and cotyledon were excised aseptically from the seedlings and cut into small pieces of 3-5 mm length.

About 4-5 pieces of hypocotyl, epicotyl and cotyledon, respectively were placed on the medium in each flasks of 100ml.

3.4 Sterilization of Laminar air flow: The bench of sterile air laminar flow was wiped clean with a swab of methylated spirit. Filtered air was blown for 5 minutes and then ultraviolet light was switched on for 30 minutes. While working, filtered air was continuously passed through the laminar air flow cabinet.

3.5 Inoculation

The explants were placed on the culture medium using sterile forceps and scalpal across the flame of sprit lamp in the sterilized laminar flow banch.

3.6 Preparation of stock solutions of basal media:

In the present investigation three culture media *viz*. MS (Murashiqe and Skoog, 1962), Blaydes (1966) and SH (Schenk and Hilderbrandt, 1972) were used. The compositions of these media are given in Table (3.1, 3.2 and 3.3). Stock solutions of macro nutrients, micro nutrients and vitamins were prepared separately by dissolving the salts as mentioned against each stock in glass double distilled water.

The stock solutions were stored in refrigeration and required quantity were pipetted out at the time of media preparation.

For the preparation of iron stock, $FeSO_4.7H_2O$ and Na_2EDTA in MS, Blaydes and SH media were dissolved separately in sufficient quantity of double distilled water. Na_2EDTA solution was boiled and then added to hot solution of $FeSO_4.7H_2O$ gently. The volume was finally made up to 100ml with double distilled water.

Table 3.1- Composition of Murashige and Skoog (1962) medium and quantity of chemicals for preparing various stock solutions.

Chemical	Concentration (mg/l)	Stock and volume	Quantity for stock (mg)	Aliquot of stock for 1 litre
NH_4NO_3	1650	A (500)	16500	50 ml
KN_3	1900		19000	
$MgSO_4.7H_2O$	370		3700	
KH_2PO_4	170		1700	
$CaCl_2.H_2O$	440	B (100)	4400	10 ml
$MnSO_4.4H_2O$	22.3	C(100)	1115	2 ml
$ZnSO_4.7H_2O$	8.6		430	
H_3BO_3	6.2		310	
KI	0.83		41.5	
$Na_2MoO_4.2H_2O$	0.25		12.5	
$CuSO_4.5H_2O$	0.025		1.25	
$CoCl_2.6H_2O$	0.025		1.25	
$Na_2EDTA.2H_2O$	37.3	D (100)	373	10 ml
FeSO4.$7H_2O$	27.8		278	
Glycine	2.0	E (100)	200	1 ml
Nicotinicacid	0.5		50	
Pyridoxine.HCL	0.5		50	
Thiamine.HCL	0.1		10	
Myo-inositol	100	F (100)	1000	10 ml

Stock solution was added in the sequence of ACEFDB. Sucrose was dissolved at the rate of 30g/l (3%). Agar was also added at the rate of 8 g/l. pH of the medium was adjusted to 5.6 to 5.8.

Table 3.2 : Composition of Blaydes (1966) medium and quantity of chemicals for preparing various stock solutions.

Chemical	Concentration	Stock and volume	Quantity for stock (mg)	Aliquot of stock for 1 litre
KH_2PO_4	300	A	3000	50 ml
KNO_3	1000	(500ml)	10000	
NH_4NO_3	1000		10000	
$MgSO_4.7H_2O$	35		350	
$Ca(NO_3)_2$	347	B (100 ml)	3470	10 ml
$FeSO_4.7H_2O$	23.6	C	236	10 ml
Na_2EDTA	32.0	(100 ml)	320	
KCL	65.0	D	3250	2 ml
KI	0.8	(100 ml)	40	
$ZnSO_4.7H_2O$	1.5		75	
$H3BO_3$	1.6		80	
$MnSO_4.H_2O$	4.4		220	
Thiamine	0.1	E	10	1 ml
Glycine	2.0	(100 ml)	200	
Pyridoxine	0.1		10	
Nicotinic acid	0.5		50	

Stock solution was added in the sequence of ACEDB. Sucrose was dissolved at the rate of 30g/l (3%). Agar was also added at the rate of 8 g/l. pH of the medium was adjusted to 5.6 to 5.8.

Table 3.3 - Composition of Schenk and Hilderbrandt (1972) medium and quantity of chemicals for preparing various stock solutions.

Chemical	Concentration (mg/l)	Stock and volume	Quantity for stock (mg)	Aliquot of stock For 1 liter
KNO_3	2500	A	25000	50 ml
$MgSO_4.7H_2O$	400	(500 ml)	4000	
$NH_4H_2PO_4$	300		3000	
$CaCl_2.2H_2O$	200	B (100 ml)	2000	10 ml
$MnSO_4.H_2O$	10.0	C	500	2 ml
H_3BO_3	5.0	(100 ml)	250	
$ZnSO_4.7H_2O$	1.0		50	
KI	1.0		50	
$CuSO_4.5H_2O$	0.2		10	
$NaMoO_4.2H_2O$	0.1		5	
$CoCl_2.6H_2O$	0.1		5	
$FeSO_4.7H_2O$	15.0	D	150	10 ml
Na_2EDTA	20.0	(100 ml)	200	
Thiamine	5.0	E	500	1 ml
Nicotinic acid	5.0	(100 ml)	500	
Pyridoxine	0.5		50	
Inositol	1000.0	F (100 ml)	10000	10 ml

Stock solution was added in the sequence of ACEFDB. Sucrose was dissolved at the rate of 30g/l (3%). Agar was also added at the rate of 8 g/l. pH of the medium was adjusted to 5.6 to 5.8.

3.7 Preparation of stock solutions of growth regulators :

(1) Auxin

2,4-D (2,4-dichlorophenoxy acetic acid)

NAA (Naphthalene acetic acid)

IAA (Indole 3-Acetic acid)

(2) Cytokinin

BAP (6- Benzyl amino purine)

Kinetin

(3) Amino acid

Proline

100ml stock solutions (1 mg/ml) of each growth regulator were separately prepared. Auxins were dissolved in a few drops of ethanol or 1N NaOH, heated slightly and gradually diluted to the required volume with double distilled water.

Cytokinins were dissolved in a few drops of 1N HCL, heated slightly and gradually diluted to the required volume with double distilled water. The amino acids were dissolved in double distilled water.

3.8 Media Preparation:

The stock solutions were mixed in the required proportion in each medium and the growth hormones were added according to requirements. Sucrose was also dissolved at the rate of 30g/l (3%). The volume was made up of 990ml and pH of the medium was adjusted to 5.6 to 5.8 by the addition of 1N NAOH or 1N HCL as required and volume was finally made up to 1 litre. Agar was added at the rate of 8 g/l of the medium and boiled in beaker till it became transparent. The media was poured into flask of 100ml capacity and plugged with non-absorbent cotton in equally distributed quantities @ 25 ml. in each flask. Then these flasks containing media were autoclaved for 20 minutes at 15 psi pressure (120°C). The autoclaved medium was kept in a laminar air flow bench for cooling and then stores in refrigerator at 4 °C-10 °C.

3.9 Plant growth regulators for callus induction:

For callus induction, different concentrations of auxin and cytokinin were tried with different basal media, *i.e.*, MS, SH and Blaydes which are as follows:

Auxin-

NAA@ 1.0,2.0,4.0 mg/l

2,4-D@ 2.0 mg/l

IAA @ 2.0,4.0 mg/l

Cytokinin-

Kinetin @ 1.0 mg/l

BAP @ 0.2 mg/l

Callus Induction

In three explants, *i.e.*, hypocotyl, epicotyl and cotyledon of eight genotypes, the callus induction was done. Best callus was found in MS medium with 2mg 2,4-D/l+1mg NAA/l+0.2mg BAP/l which was also better in colour and texture.

Recording of observation

The observations of callus induction were recorded on the following aspects:

(a) Callus induction frequency of explants

The number of explants inoculated and the number of explants callused per replication, 20 days after inoculation were counted. Callus induction frequency was completed as

$$\frac{\text{Number of explants callused}}{\text{Number of explants inoculated}} \times 100$$

(b) Callus colour

The callus colour was recorded on a visual scale from 1 to 4 as given below:

1- Brown callus

2- Dark yellow callus

3- Light yellow callus

4- Green callus

(c) Callus texture

The callus texture was recorded on a visual scale from 1 to 3 as given below:

1-Watery

2-Granular

3-Friable

Incubation of cultures-

For initial callus induction and subsequent callus maintenance, all cultures were kept in dark at 25 ± 2^0C.

Subculturing

Callus was maintained by successive subcultures at a regular interval of 20-21 days on the MS medium with 2,4-D @ 2mg/l, NAA@ 1mg/l, and BA @ 0.2mg/l of medium. The observation of the callus colour and texture were recorded.

Fresh and Dry weight of callus:

The fresh weight and dry weight of callus was taken in all the 8 genotypes (C-10, IG-1212, IL-75, A-3, LLC-9, LLC-3, Anand-2 and AL-95-12) from 3 explants (Hypocotyl, epicotyl and cotyledon). The explants were inoculated in MS with 2mg 2,4-D/l+1mg NAA/l+ 0.2mg BAP/l combination of media for callus induction. For each genotype, 4-5 bits of each explant were inoculated in 4 flasks (100ml) after recording the initial weight of explants inoculated in each separate flask. Flasks were incubated in dark. After 20 days, out of 4 flask the callus of one flask was taken out, fresh weight was recorded, then covered with aluminium foil and kept in oven at 60°C for 3-4 days for drying and then the dry weight of callus were taken. Similarly, one flask of each at 20 days interval up to 80 days, were used to record fresh and dry weight of calli. The calli were regularly subcultured in the same medium every 20 days till their weight were recorded.

3.10 Optimization for *in vitro* regneration :

The calli were subcultured on MS and SH media with various combinations of plant growth regulators. They were incubated at 25 ± 2 ^{0}C temperature and 14 hours light (2500 - 3000 lux light intensity) + 10 hours dark photoperiod sequentially. The regeneration studies were conducted following two pathways (A) Morphogenetic response of calli directly on regeneration media and (B) Morphogenetic response of calli on sequence of regeneration media following auxin shock treatment.

Auxin shock treatment

Calli from different explants of the different genotypes were transferred on SH medium containing 11.0 mg/l 2,4-D + 1.0 mg/l kinetin (auxin shock treatment medium) for auxin shock for four days, following Romagnoli *et al.* (1966).

After auxin shock treatment the sequence of regeneration mediums followed for induction of regeneration from the calli derived from different explants of all the genotypes under study are given below:

Regeneration media sequenc

SHKI :

SH + 4.0 mg/l kinetin + 1.0 mg/l IAA

↓

SH + 8.0 mg/l kinetin + 1.0 mg/l IAA

↓

SH + 2.0 mg/l kinetin

↓

Plain SH

MSKI :

MS + 4.0 mg/l kinetin + 1.0 mg/l IAA

↓

MS + 8.0 mg/l kinetin + 1.0 mg/l IAA

↓

MS + 2.0 mg/l kinetin

↓

Plain MS

SHPKI :

SH + 15.0 mg/l proline

↓

SH + 4.0 mg/l kinetin + 1.0 mg/l IAA

↓

SH + 8.0 mg/l kinetin + 1.0 mg/l IAA

↓

SH + 2.0 mg/l kinetin

↓

Plain SH

MSPKI :

SH + 15.0 mg/l proline

↓

MS + 4.0 mg/l kinetin + 1.0 mg/l IAA

↓

MS + 8.0 mg/l kinetin + 1.0 mg/l IAA

↓

MS + 2.0 mg/l kinetin

↓

Plain MS

3.11 Cellular and histological observations:

Some characters related to cell morphology were observed in regenerating as well as non regenerating cells so as to find the types of cell masses undergoing regeneration. Smears from the portions of fresh calli, representing non regenerating and regenerating calli were made on the cleaned microslides and stained with 1.0% acetocarmine followed by gentle heating on a sprit lamp for staining of nucleus and cytoplasm.

Fresh calli exhibiting regeneration potential were fixed in Carnoy's aceto-alcohol (acetic acid and alcohol, 3:1 v/v) for 24 hours, washed with 70% alcohol and subsequently stored in 70% alcohol. The samples for study were selected and passed through tertiary butyl alcohol dehydration –infiltration series and embedded in petro-wax (Johansen, 1940).

The sections were cut at 10μ thickness with the help of a micrtome (WESWOX, India) and spread on thoroughly cleaned microslides. Cleaning the slides was done by putting them for overnight in chromic acid solution, thereafter washing thoroughly with water and lastly with 95% ethanol, and dried. Mayer's adhesive (Johansen, 1940) was used for affixing the sections to the slides and highly diluted gloy solution was used for floating the adhesive smeared slides to facilitate spreading of the sections.

The microtomed sections were dewaxed with xylene and brought to water through graded alcohol series. Northem's variation of Foster's tannic acid-ferric chloride, safranin and fast green stain series (Johansen, 1940) was used for staining of the sections of calli and regenerating tissues. The sections were cleared with xylene and mounted in DPX mountant.

Microscopic observations and photographs of the sections were taken with the help of upright trinocular compound microscope Opti - phot-2 with photomicrographic attachment and automatic exposimeter HFX-DX (Nikon, Japan).

3.12 Growth regulators for root differentiation:

For root differentiation, different concentration of auxins and cytokinins and some adjuvant such as charcoal etc. were used which are as follows-

RM1 - MS+ 0.05 mg/l kinetin +2.0 mg/l NAA +2.0 mg/l IAA

RM2 - MS+ 0.05 mg/l kinetin +2.0 mg/l NAA +2.0 mg/l IAA +3.0 g/l charcoal

RM3 - MS+ 0.05 mg/l kinetin +2.0 mg/l NAA +5.0 mg/l IAA

RM4 - MS+ 0.05 mg/l kinetin +2.0 mg/l NAA +5.0 mg/l IAA +3.0 g/l charcoal

RM5 - SH+ 0.05 mg/l kinetin +2.0 mg/l NAA +2.0 mg/l IAA

RM6 - SH+ 0.05 mg/l kinetin +2.0 mg/l NAA +2.0 mg/l IAA + 3.0 g/l charcoal

RM7 - SH+ 0.05 mg/l kinetin +2.0 mg/l NAA +5.0 mg/l IAA

RM8 - SH+ 0.05 mg/l kinetin +2.0 mg/l NAA +5.0 mg/l IAA + 3.0 g/l charcoal

3.13 Hardening:

Small plastic pots were filled with autoclaved soilrite and soaked with 1/4 strength MS media containing no growth regulators and sucrose. The regenerated plants were taken out the flasks, their roots were washed with sterile water, planted in soilrite in the plastic pots and covered with polythene bag (to maintain the moisture inside). These were kept in tray filled with sterile tap water. The tray was kept in incubation room in 16 hours light and 8 hours dark for 6-7 days. During this incubation period, the potythene bags were removed from all the plants after 3 days. Subsequently the surviving plants were transferred to larger plastic pots containing soil, sand and FYM (1:1:1 v/v) and kept in diffused sunlight at room temperature for 8-12 days. Then these plants containing pots were transferred to the field. After 7-8 days, when the plants were sufficiently acclimatized, they were planted in the experimental plots in the field.

3.14 Somaclonal variation:

42 somaclones of LLC-3 genotypes were grown in field along with parent plant.

Morphological observations

The observation on morphological traits were taken in 42 somaclones developed from LLC-3 genotype and 5 parent plants. The data on various morphological characters, *viz.*, (1) Plant height, (2) branches/ plants, (3) number of nodes, (4) length of internodes, (5) stem girth, (6) fresh leaf weight, (7) fresh stem weight, (8) fresh leaf/stem weight ratio, (9) total green weight, (10) dry leaf weight, (11) dry stem weight, (12) total dry weight, (13) dry matter content, (14) flower length, (15) flower width and (16) flower size were recorded.

All the somaclones and parent plants were cut at the onset of rabi season and the plants flowered in about 2 months from the date of cutting. The recording of observations started 10 days after flowering. Then plants were cut from the base and fresh weight of leaves and stem were taken separately. These were kept in different envelopes for 3-4 days at room temperature and then kept in oven for drying at 60°C for 48 hours. Then dry weight of leaf and stem was recorded. Observation were recorded as follows-

1. *Plant height :* Height of plant was recorded from ground level to the tip of flower on the main branch in centimeters.
2. *Branches/ plant:* Number of branches on the main stem were recorded from the ground level of the plant.
3. *Number of nodes:* Number of nodes on the main axis were counted.
4. *Length of internodes :* Length of internodes was measured on the main stem in centimeters.
5. *Stem girth :* Girth of the main stem was recorded in millimeters with the help of vernier calipers at three places of main branch.
6. *Fresh leaf weight :* Plants were harvested at the base and all the leaves from the plants were collected and weighted in grams.
7. *Fresh stem weight :* Plants were harvested at the base and entire stem from the plants were collected and weighted in grams.
8. *Fresh leaf/stem weight ratio :* The ratio was calculated by dividing the fresh leaf weight by fresh stem weight.

9. *Total green weight :* Total green leaf and stem were weighted in grams per plant.
10. *Dry leaf weight :* Fresh leaves were dried in oven at 60°C for 48 hours and weighed in grams.
11. *Dry stem weight :* Fresh stem were dried in oven at 60°C for 48 hours and weighed in grams.
12. *Total dry weight :* Total dry leaf and stem were weighed in grams to get the total dry weight per plant.
13. *Dry matter content :* This was calculated as per cent total dry weight over total fresh weight.
14. *Flower length :* The length of the petal of the flower was measured in centimeters from base of the tip.
15. *Flower width :* The width of the petal of the flower was measured in centimeters.
16. *Flower size :* Size (LxW) of 4 flowers in main branch of each plant was recorded.

Isozyme:

Polyacrylamide gel electrophoresis (PAGE) method was used for isozyme analysis

Extraction buffer- composition of extraction buffer in 100 ml

1. Tris HCL - 100 mM (pH-7.0 adjusted by concentrated HCL)
2. β-mercaptoethanol - 10mM or 75µl
3. Sucrose - 10 %

Extraction

Young leaves of 6 somaclones and 1 plant of LLC-3 genotype were collected from the field in icebox. 33g of leaves were taken from each sample and were ground using pre- cooled pestile and mortor, in cold (in a tray filled with ice) in 600 µl extraction buffer. The samples were collected in eppendorf tube with the help of spatula. Then samples were centrifuged at 4°C temperature and 10,000 rotations per minute (r.p.m) for 20 minutes. After 20 minutes supernatant was collected and the

pellet was discarded. Then supernatant was kept in deep freezer (at 40°C) for isozyme analysis.

Electrophoresis

Discontinuous phase (vertical) polyacrylamide gel electrophoresis.

Resolving gel buffer – Composition of resolving gel buffer (R.G.B.)

1. Tris HCL- 1.5 M (pH 8.8 adjusted by concentrated HCL) in 100ml.

Staking gel buffer-Composition of staking gel buffer (S.G.B.)

1. Tris HCL- 0.5 M (pH 6.8 adjusted by concentrated HCL) in 100 ml.

Stacking gel (for 10 ml)-

1. Water- 11.5 ml
2. R.G.B.- 2.5 ml
3. Ammonium per sulphate- 1.0ml
4. TAMED- 5.0 µl

Resolving gel (For 20 ml) -

1. Water – 20 ml
2. R.G.B.- 2.5 ml
3. Acrylamide- 5.0 ml
4. TAMED - 10µl

Electrophoresis

Discontinuous phase vertical polyacrylamide gel electrophoresis was done using mini protean xi II apparatus (BIORED, U.S.A.).

Running conditions of gel

Tank Buffer- Composition of tank buffer in 1000 ml and pH 8.3 maintained by glycine and tris HCL.

1. Tris HCL- 3 g
2. Glycine- 14.4 g

The electrophoresis was carried out at 50 volt electric potential for 15 minutes followed by 100 and 150 volts till the end of the run.

Gel staining-

Esterase

1. 60 mg Of alpha naphthyl acetate in 5 ml Of 60% acetone
2. 20 ml Of NaH_2PO_4 (0.624)
3. 10 ml Of Na_2HPO_4 (0.284)
4. 20 ml of distilled water.
5. 80 mg Of fast blue RR salt dissolved in 3 ml Of acetone

1, 2, 3 and 4 were mixed to which gel are added and incubated for 10 minutes and solution 5 was added and destained with 40 ml methanol+ 40ml water+ 8ml Ethanol +4ml acetic acid.

Glucose 6Phosphate dehydrogenase (G6PDH)

1. 0.05 M Tris HCL - 50 ml (pH 8.0)
2. 50 mg Of glucose 6 phasphate di sodium salt

Staining solution in 1 ml of the above buffer

1. Nicotanimide adenine di nucleotide phosphate (NADP) - 5 mg
2. Methylthiazoletetrazolium (MTT) - 10 mg
3. Phenazine methosulphate (PMS) - 2mg
4. Magnesium chloride- 50 mg

Incubate the gel in buffer with substrate and add staining solution. Then destained with 50 per cent glucerol.

Phosphoglucomutase (PGM)

0.01 M Tris HCL - 50 ml (pH 7.5)

Glucose 1 phosphate - 80 mg

Glucose 6 Phosphate dehydrogenase - 6µl.

Staining solution

Nicotanimide adenine dinucleotide phosphate (NADP) - 10mg

Methylthiazoletetrazolium (MTT) - 15mg

Phenazine methosulphate (PMS) - 1mg

Adenosin tri phosphate (ATP) - 15 mg

Magnesium chloride (MgCl2) - 175mg

Gel was incubated in buffer with substrate and enzynme for 10 minutes and staining solution is added and destained with 50 per cent glycerol.

Phosphogluco isomerase (PGI)

0.1M Tris HCL - 50 ml (pH- 7.5)

Fructose 6 phosphate di sodium salt- 20 mg

Glucose 6 phosphate dehydrogenase - 6µl

Staining solution

Nicotanimide adenine di nucleotide phosphate (NADP) - 10 mg

Methylthiazoletetrazolium (MTT) - 10mg

Phenazine methosulphate (PMS) - 2mg

Magnesium chloride (MgCl2) - 150mg

Gel is incubated with buffer with substrate for 10 minutes and staining solution is added and destained with 50 per cent glycerol.

Aspartate Amino Transferase (AAT)

α ketoglutaric acid - 35mg

L-aspartic acid - 130mg

Poly vinyl pyrrolidone (PVP MW 40,000) - 500mg

Ethylene Diamine Tetra Acetic Acid Disodium salt (EDTA) - 50mg

Disodium hydrogen arthophosphate anhydrous (Na2HPO4) - 1.4g

100ml volume make-up with distilled water and pH adujested by aspartic acid and α-keto gluteric acid.

Staining solution

Fast blue BB salt - 100mg for 100ml

Superoxide dismutase (SOD)

50mM Tris HCL - 50ml (pH adjusted by concentrated HCL)

Riboflavin - 5mg

Ethylene diamine tetra acetic acid disodium salt (EDTA)- 2mg

Methylthiazoletetrazolium (MTT)- 20mg

Gel was kept for 20 minutes in dark and 15 minutes in strong light.

Visualization of gels

Gels was observed in white light transilluminator and zymograms were prepared.

RAPD

Genomic DNA isolation :

Genomic DNA was extracted from somaclones and their parent according to Dellaporta *et al.* (1983) with slight modification, using CTAB method. 2 gm of young leaves were collected and were frozen in liquid nitrogen. It was powered using pestle and morter and was transferred to oakridge tubes. 10 ml of extraction buffer (100 mM Tris HCL, pH 8.0, 20 mM EDTA, pH 8.0, 1.4 M NaCl, 2% w/v CTAB and 1% w/v polyvinylpyrrolidone mw, 40,000) was added to the tubes and were incubated at 65°C for 30 minutes followed by addition of equal volume of chloroform: isoamyl alcohol (24:1). They were later centrifuged at 10,000 rpm 5 minutes at 4°C and collected the upper aqueous layer followed by addition of 1/10 volume of CTAB /NaCl solution (0.7 M NaCl and 10 % CTAB). Further, an equal volume of chloroform: isoamyle alcohol (24:1) was added to these tubes and were mixed by inverting them. They were again centrifuged at 5,000 rpm for 5 minutes at 4°C. Upper aqueous layer was collected and an equal volume of CTAB/precipitation solution (50 mM Tris HCL, Ph 8.0, 10 mM EDTA, pH 8.0 and 1% CTAB) was added. The tubes were centrifuged at 4000 rpm at 4°C for 5 minutes. Pellets were dissolved in TE buffer (10 mM Tris Cl, pH 8.0, 0.1 mM EDTA, pH 8.0 and 1M NaCl) and DNA was precipitated into 0.6 volume of cold isopropanol for at least one hour at -20 °C. Finally the tubes were centrifuged at 7000 rpm for 5 minutes at 4°C. Pellets obtained were washed in 70 % ethanol and were resuspended in 100 ml of TE buffer (10 mM Tris HCL pH 7.4 and 1 mM EDTA, pH 8.0).

DNA was further purified, after treatment with 2µl of Rnase (10 mg/ml) for 100 µl of DNA it was incubated at 37°C for one hour. DNA was phenolized and centrifuged at 5,000 rpm for 15 seconds at 4°C and collected upper aqueous layer followed by precipitation by addition of 1/10 volume 3 M sodium acitate, pH 5.2. They were centrifuged at 10,000 rpm for 5 minutes at 4°C, taken out pellet and was washed in 70 % ethanol and vaccum dried and the purified DNA was redissoloved in TE (10 mM, pH 7.4 and 1 mM EDTA, pH 8.0). The quality and quantity was checked by electrophoresis on agarose gel and quantitated by spectrophotometric analysis.

Purified DNA was run on 0.8% agarose gel on horizontal gel electrophoresis system (Bio-Red), using 1X TAE buffer.

Spectrophotometric estimation was used to quantify the amount of genomic DNA, to evaluate its purity (A_{260}/A_{280} values) and the adjust samples to a concentration of 20ng/µl.

Spectrophotometer analysis of DNA :

$$\mu g/\mu l \text{ of DNA} = \frac{OD_{260} \times \text{dilution factor}}{1000} \quad X\ 50$$

S.N	OD_{260}	Dilution factor	DNA quantity in (μg/μl)
1	0.357	720	$\frac{0.357 \times 720 \times 50}{1000}$
2	0.323	720	$\frac{0.323 \times 720 \times 50}{1000}$
3	0.304	720	$\frac{0.304 \times 720 \times 50}{1000}$
4	0.154	720	$\frac{0.154 \times 720 \times 50}{1000}$
5	0.069	720	$\frac{0.069 \times 720 \times 50}{1000}$
Parent	0.095	720	$\frac{0.095 \times 720 \times 50}{1000}$

Dilution of sample DNA to 20ng/μl :

DNA was diluted with water to get a concentration of 20ng/μl.

Buffers and Reagents :

CTAB extraction solution –

100 mM Tris Cl, pH 8.0

20 mM EDTA, pH 8.0

1.4 M NaCl

2 % w/v CTAB

1 % PVP, 40,000

CTAB/NaCl solution –
0.7 M NaCl
10 % CTAB

CTAB ppt. Solution –
50 mM Tris Cl, pH 8.0
10 mM EDTA, pH 8.0
1 % CTAB

High salt buffer –
10 mM Tris Cl, pH 8.0
0.1 mM EDTA, pH 8.0
1 M NaCl

TE buffer –
10 mM Tris Cl, pH 7.4
1 mM EDTA, pH 8.0

50X TAE – quantity for 1 litre
242 gm TrisHCL, pH 8.0
57.1 ml Glacial Acetic Acid
100 ml, 0.5 M EDTA, pH 8.0
make up volume to 1 litre and adjust pH to 8.0

6X gel loading dye (stored at room temperature) –
0.25 % Bromophenol Blue (BPB)
0.25 % Xylene Cyanol FF
40 % (w/v) Sucrose

Ethidium Bromide – 10 mg/ml
Dissolved 100 mg ethidium bromide in 100 ml sterile water and stored at 4°C, wrapped with aluminum foil.

Rnase – (10 mg/ml)

Quantity for 1 ml

Rnase A = 10 mg

5 M sodium acetate, pH 5.2 = 5µl

1 M Tris HCL = 10 µl

All these were added and heated to 100°C for 15 minutes followed by cooling slowely to room temperature. The pH 7.5 was adjusted with 1 M Tris HCL and the aliquot was taken into 1 ml eppendorf tube and store at -20 °C.

PCR amplification conditiond :

The PCR conditions used were similar to those described by Williams et al. (1990), with slight modification. The reaction was done in a DNA thermal cycler (Perkin Elmer, Version 480) programmed for 2 minutes at 94°C (denaturation) and 40 cycles of 94°C for 1 minute (denaturation), 1 minute at 36°C (annealing) and 2 minutes at 72 °C (extension) and last 5 minutes at 72 °C (extension).

PCR amplification were performed in 25µl total volume reactions containing 40 ng of genomic DNA, 1X reaction buffer (10 mM Tris HCL, pH 8.3, 50 MM KCL, 2 mM $MgCl_2$, 0.001 % gelatin), 100µM each of dATP, dCTP, dGTP and dTTP, 4 mM $MgCl_2$, 0.5 µM Primer, 1 unit Taq polymerase and sterilized double distilled water. The reaction mixture was overlayed with 20µl of miniral oil. A total of 15µl of the reaction mixture was loaded into the wells and products were separted by electrophoresis in horizontol gel system (Bio-Red), using 1 X TAE buffer on 1.4 % agarose gel, containing 0.15µg/ml ethidium bromide. The gel was run at ~60 volt for 3 hours and bands were visualized under UV transilluminator.

PCR cycle fragment :

i. 94 °C for 2 minute

ii. 40 cycles

94 °C for 1 minute

36 °C for 1 minute

72°C for 2 minute

iii 72°C for 5 minute

Primer Screening :

Random decamer primers (Operon Technology Inc., Calif., Alameda, USA) of the series of J, C, M and E have been used. Out of 11 primers screened, following six primers were found to give good results.

Primer sequence :

1. OPJ-01 – 5'CCCGGCATAA3'
2. OPJ-04 – 5'CCGAACACGG3'
3. OPJ-05 – 5'CTCCATGGGG3'
4. OPJ-06 – 5'TCGTTCCGCA3'
5. OPM-04 – 5'GGCGGTTGTC3'
6. OPE-01 – 5'CCCAAGGTCC3'

PCR reagent

Componants	Quantity added	Final	Master mix (6)
10 X buffer	2.5µl	1 X	15
dATP (10 mM)	0.25µl	0.1 mM	1.5
dCTP (10 mM)	0.25µl	0.1 mM	1.5
dTTP (10 mM)	0.25µl	0.1 mM	1.5
dGTP (10 mM)	0.25µl	0.1 mM	1.5
$MgCl_2$ (25 mM)	3.5µl	3.5 mM	21
Primer (25 µM)	0.5µl	0.5 µM	3.0
Taq Polymerase (3 U/µl)	0.3µl	1 unit	1.8
DNA (20 ng/µl)	2 µl	40 ng/25µl	_
Distilled water	15.2	_	91.2

Estimation of chemical constituents

Dry matter, C.P and ash contents of the samples were determined as per the method of AOAC (1990). Cell wall contents / fiber fractions (NDF, ADF, Cellulose and Lignin) were analysed following the method of Goering and VanSoest (1970). Analysed constituents were expressed on per cent dry matter basis.

Drying of samples

Representative samples were dried in hot air oven at 70°C for 48-72 hours. The dried sample weight was taken as DM content.

Preparation of samples

The dried samples were ground through 2mm sieve using willey will. These ground samples were to the analysis of different nutritional constituents.

Crude Protein

For CP estimation one g ground dried sample was put in the digestion tube. To the digestion tube 8-10 g of digestion mixture (mixture of $CuSO_4$ and K_2SO_4 in the ratio of 9:1) was added. Digestion of the samples was done by adding 20ml concentrations H_2SO4 for 4-6 hours till no black particle was left and it became transparent. The digested samples were transferred to this volumetric flask and volume was made up to 50 ml with distilled water. After shaking contents of volumetric flask 10 ml of aliquot was taken and distilled in Micro Kjeldahl distillation apparatus 40 per cent NaOH. Released ammonia was collected in a beaker containing 20 ml of 4 per cent boric acid mixed with indicator (12 ml of methyl red 0.1% and 6ml of bromocresol green of 0.1%). The color of indicator changed from red to green and released ammonia gas, which was absorbed by boric acid. Then ammonium borate was titrated with standard solution of sulphuric acid and finally "N" (Nitrogen) percentage was calculated as-

N/100 H_2SO_4 = 0.00014 g

$$\% \text{ Crude protein} = \frac{V \times 0.00014 \times D \times 100 \times 6.25}{W \times A}$$

Where,

V = Titer value

D = Dilution factor (volume made in volumetric flask)

W = Weight of sample

A = Aliquot taken

Since average nitrogen content of most of the protein is 16%

$$1\text{g Nitrogen} = \frac{100}{16}$$

$$= 6.25 \text{ g protein}$$

Neutral detergent fibre (NDF):

Reagent

(i) Neutral detergent solution:

It was prepared by dissolving 18.61g disodium ethylene diamine tetraacetate dihydrate (EDTA) and 6.81g sodium borate decahydrate in about 500 ml of distilled water by heating on a boiling water bath. Then 30g sodium lauryl sulphate dissolved in about 200 ml of hot distilled water was added. To this 4.56g anhydrous disodium hydrogen phosphate dissolved in about 100 ml of hot distilled water was mixed. After cooling, 10 ml of 2- ethoxyethanol was added to the mixture and volume was made up to one litre.

(ii) Acetone

(iii) Sodium sulphite

Method

One g dried sample was kept in 600 ml beaker and 100 ml of neutral detergent solution. 2 ml decahydronaphthalene and 0.5 g sodium sulphite were added. The contents were boiled for 5-10 mintues. Boiling was adjusted and refluxed for one hour. The extracted material was filtered through a tared Gooch crucible under vacuum. Any residue left in the beaker was transferred to the Gooch crucible with hot distilled water (90-100°C) and filtered again. Washing procedure was repeated

twice with hot water and final washing was done with acetone till the filterate was free from color. The crucible was kept in an oven maintained at 80°C for overnight, crucible were kept in dessicator and weighted. The gain in the weight of the crucible was expressed as neutral detergent fibre.

$$\% \text{ Cell wall constituents (NDF)} = \frac{\text{Weight of crucible+cell wall constituent-weight of crucibl}}{\text{weight of sample}} \times 100$$

Cell contents = 100 – NDF (%)

Acid detergent fibre (ADF):

Reagents

(i) Acid detergent solution

This solution was prepared by dissolving 20g cetyltrimethyl ammonium bromide (CTAB) in one litre of 1 N sulphuric acid with gradual shaking.

(ii) Acetone

Method

One g oven dried sample was kept in 600 ml beaker and 100 ml of acid detergent solution. The contents were boiled for 5-10 minutes. Boiling was adjusted and refluxed for one hour. The extracted material was filtered through a tared Gooch crucible under vacuum. Any residue left in the beaker was transferred to the Gooch crucible with hot distilled water (90-100°C) and filtered again. This washing procedure was repeated twice with hot water and final washing was done with acetone till the filterate was free from colour. The crucible was kept in an oven maintained at 100°C for overnight, cooled in a dessicator and weighted again. The gain in the weight of crucible was expressed as acid detergent fibre.

Hemicellulose:

The hemicellulose content was calculated by substracting the values of acid detergent fibre from neutral detergent fibre.

Cellulose and lignin:

Reagents

(i) 72% sulphuric acid by weight

(ii) Acetone

Method

The crucible with ADF contents were treated with 72 per cent H_2SO_4 and stirred with a glass rod to a smooth paste, breaking all lumps. The crucible was again half filled with 72 per cent H_2SO_4, stirred regularly at an interval of 1hour and kept for 3hour at 20-30°C. The excess of sulphuric acid was filtered off under vacuum and contents were washed with hot water until free from the acid. The glass rods were also rinsed with hot water. The crucibles were kept in an oven at 100°C for overnight, cooled in a dessicator and weighted again. The loss in the weight of crucible was expressed as cellulose contents.

$$\text{Cellulose (\%)} = \frac{\text{ADF (\%) – After 72 \% sulphuric acid}}{\text{Weight of sample}} \text{ x100}$$

Crucibles were then transferred to a muffle furnace and ignited at 550°C for 3hours. The crucible were then cooled in a dessicator and weighted again. The loss in the weight was taken as lignin content.

$$\text{\% acid detergent lignin} = \frac{\text{Weight of crucible and lignin-weight of crucible and ash}}{\text{Weight of sample}} \text{ X 100}$$

Availability index

AI values of leaf and stem fractions were estimated adopting the procedure of Van Soest and Moore (1965).

$$\text{\% AI} = \left[100 - \left(\frac{\text{lignin x 100}}{\text{NDS}} \right) \right]$$

3.15 Statistical analysis

Tissue culture analysis : The statistical methods suggested by Compton (1994) were followed. Analysis of variance for separate effects of media, growth regulators and media adjuvants for callus induction and regeneration with different genotypes were performed using factorial completely randomized design (CRD) with unequal

number of replications. Each treatment was replicated at least five times with 5 to 6 explants per replication.

Isozyme analysis : The band pattern of isozymes in six distinct somaclones were selected for studying somaclonal variation. The zymogrames were recorded and the isozyme bands were scored as present (as 1) or absent (as 0) at every loci to derive the electrophoretic phenotype (EP) for each sample. A matrix of simple matching coefficient was generated using the zymogram data and a phenogram was generated with the unweighed pair-group method using an arithmetic average (UPGMA) according to Sneath and Sokal (1973).

Morphological analysis : Morphological analysis was done according to Fukui (1986).

$M \pm \delta \sqrt{(F (N+1)/N)}$

M – mean of control

δ - S.D of control

F – table F valus (n1 =1, n = N-1)

N – no. of plants in control

$$SVF\ \% = \frac{\text{\# of variants in SC1}}{\text{Total number of plants in SC1}} X100$$

Callus colour and texture :

The visual score given for callus colour and texture were analyzed using a non parameteric Kruskal-Wallis statistic (Hollander and Wolfe, 1973) since these data were recorded on ordinal scale. Kruskal-Wallis statistic was computed as given below.

Step1

All the observation in K groups were ranked in a single series assuming ranks from 1 to N (tied observations were assigned the value of the average of the tied ranks).

Step 2 :

Kruskal-Wallis statistic was calculated as follows :

$$KW = \frac{12}{N (N+1)} \sum_{j=1}^{k} nj\ (Rj-R)^2$$

Where

K = Number of groups or samples

n_j = Number of cases in the j^{th} group (replication)

N = Number of cases in the combined samples (the sum of the n_js)

R_j = Average of the rans in the j^{th} group

R = A (N+1)/2 = the average of the ranks in the combined sample (the grand mean) and the summation is across K samples.

The significance of the calculated KW was determined as exceeding the critical chi-square distribution at (K-1) degree of freedom. The significance of the individual pairs of differences was tested by the following in quality:

$$|R_u - R_v| \geq Z\alpha \mid K(K-1) \left[\frac{N(N+1)}{12}\right]^{1/2} \left[\frac{1}{nu} + \frac{1}{nv}\right]^{1/2}$$

where

$|Ru-Rv|$ = Absolute difference between means of ranks of two groups U & V

$Z\alpha \mid K(K-1)$ = The abscissa value from the unit normal distribution above which lies $\alpha / K(K-1)$ per cent of the distribution

n_u & n_v = Number of replications in U & V group, respectively

N = Number of cases in the combined sample

The significance of interaction effects between genotypes and media could not be worked out because Kruskal-Wallis statistic described above holds good for completely randomized design. Analysis was made for each genotype separately to find the best of 7 callusing media (1c to 7c) for the callus colour, callus texture of that genotype. For comparing the effect of genotype on colour and texture of callus, replication wise averages were made over 7 callusing media for each genotype.

The overall effects of media on callus colour and texture were compared by ranking replication wise averages over eight genotypes. The significance of these effects were separately tested by the Kruskal Wallis statistic as described above.

EXPERIMENTAL RESULTS

4. *RESULTS*

The results in the present study have been grouped under following two groups of experiments -

First major group : Effect of genotypes and explants for callus induction, callus quality (colour and texture), callus growth and *in vitro* morphogenetic response (Regeneration).

Second major group : Evaluation of somaclones and their comparison with the parent material for somaclonal variation.

4.1. Effect of genotypes and explants for callus induction, callus quality (colour and texture), callus growth and *in vitro* morphogenetic response (Regeneration)

4.1.1. Culture media

Effect of different tissue culture media on callus induction frequency in lucerne was determined. For this experiment, the mature seeds of LLC-3 genotype of lucerne were germinated aseptically on MS basal medium. The first response of the seeds of lucerne on any tissue culture medium was their germination and initial fast growth of the seedlings differentiating into various organs, prior to callus formation. Hence, the hypocotyls, epicotyls and cotyledons from seven days old seedlings were harvested, cut into small pieces of 3-5 mm length and inoculated aseptically on three different basal media, namely SH, MS and Blaydes supplemented with 3.0 mg/l 2,4-D and 0.5 mg/l kinetin for evaluating their performance for callus induction frequency (per cent explant responded) and callus quality (callus colour and texture) response. Callus quality was recorded on a visual score scale (1 to 4). The data are presented in Table-4.1 and 4.2.

The mean callus induction frequency was recorded maximum on MS medium (86.66 %) followed by SH medium (78.6 %), while Blaydes medium showed minimum

callus induction response (65.33 %). The overall maximum callus indu response was observed on MS medium from hypocotyl explant (92.0 %) foll by the callus induction response from the cotyledon explant equally both on M SH media (90.0 %). The overall minimum callus induction response was obs on Blaydes medium from cotyledon explant (64.0 %). Hypocotyl explant (Pl Fig.1) exhibited maximum callus induction on MS medium (92.0 %) follow SH medium (80.0 %) and the minimum callus induction from this explant observed on Blaydes medium (62.0 %). The epicotyl explant (Plate-1, Fig.2) exhibited maximum callus induction on MS medium (78.0 %) followed b medium (66.0 %) and the minimum callus induction from this explant was obs on Blaydes medium (70.0 %). The cotyledon explant (Plate-1, Fig.3) showed c induction response equally well on SH and MS media (90.0 %) and it was 65.. on Blayde's medium.

These media also exhibited differences in callus induction response from diff explants. SH medium (Plate-2, Fig.2) showed best response of callus induction cotyledon explant (90.0 %), followed by hypocotyl (80.0 %) and the mini m response was observed from epicotyl explant (66.0 %). MS medium (Plate-2, F 1) showed best callus response from hypocotyl explant (92.0 %) which as comparable with that from cotyledon (90.0 %) and the minimum response as observed from epicotyl explant (78.0 %).

Blayde's medium (Plate-2, Fig.3) exhibited best callus induction frequency from epicotyl (70.0 %) followed by cotyledon (64.0 %) and hypocotyl (62.0 %), which were comparable. Cotyledon explant performed best (81.33 %) followed by hypocotyl (78.0 %) and epicotyl (71.33 %) in terms of callus induction frequency on all the three culture media. On the basis of callus induction frequency MS medium was found to be the best followed by SH medium.

On the basis of visual observation on these callus quality parameters, there was no overall difference in the callus texture among all the three media, but for callus colour. MS medium was found best (3.67) followed by SH medium (3.5). The mean

performance of Blaydes medium was lowest for this parameter (2.67). The cotyledon explant exhibited best quality of callus in terms of callus colour (4.0) followed by epicotyl (3.0) and hypocotyl (2.8) explants. However, highly significant differences were observed for callus colour from epicotyl explant.

Table 4.1 - Effect of different media and explants on callus induction frequency

Media	Hypocotyl	Epicotyl	Cotyledon	Mean
SH	80.00	66.00	90.00	78.66
MS	92.00	78.00	90.00	86.66
Blaydes	62.00	70.00	64.00	65.33
Mean	78.00	71.33	81.33	-

Test - Fvalue

Media - 0.0517

Explant - 0.4942

Interaction - 0.6257

Table 4.2 - Effect of different media and explants on callus colour and texture

Media	Hypocotyls		Epicotyl		Cotyledon		Mean	
	colour	texture	colour	texture	Colour	texture	colour	texture
SH	3.0	2.0	3.5	2.0	4.0	2.0	3.5	2.0
MS	3.5	2.0	3.5	2.0	4.0	2.0	3.67	2.0
Blaydes	2.0	2.0	2.0	2.0	4.0	2.0	2.67	2.0
Mean	2.8	2.0	3.0	2.0	4.0	2.0	-	-
Kw	3.91	0.69	9.87	0.02	0.56	0.00	-	-

Callus Induction

Plate - 1

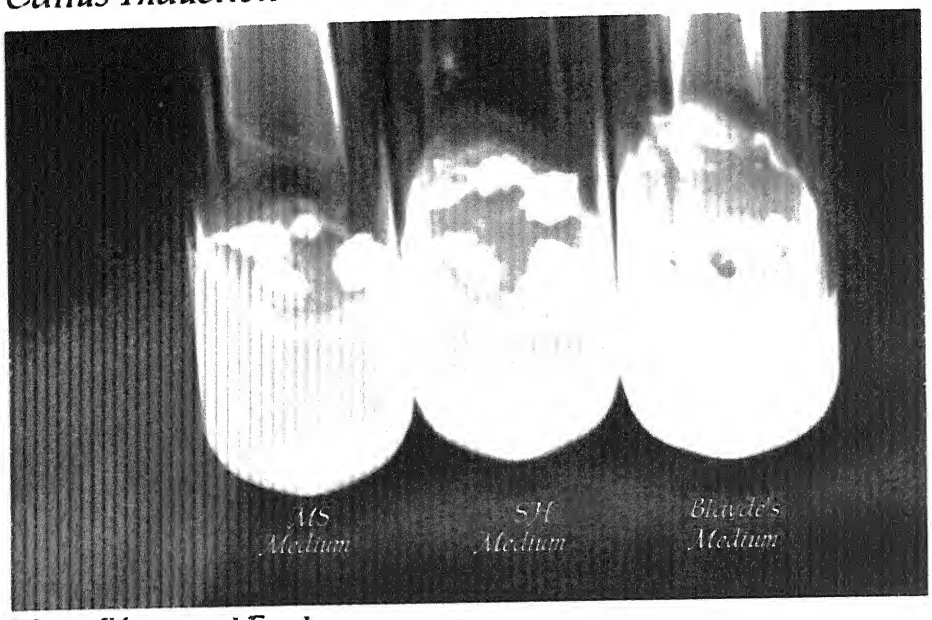


Fig.1 - Hypocotyl Explant

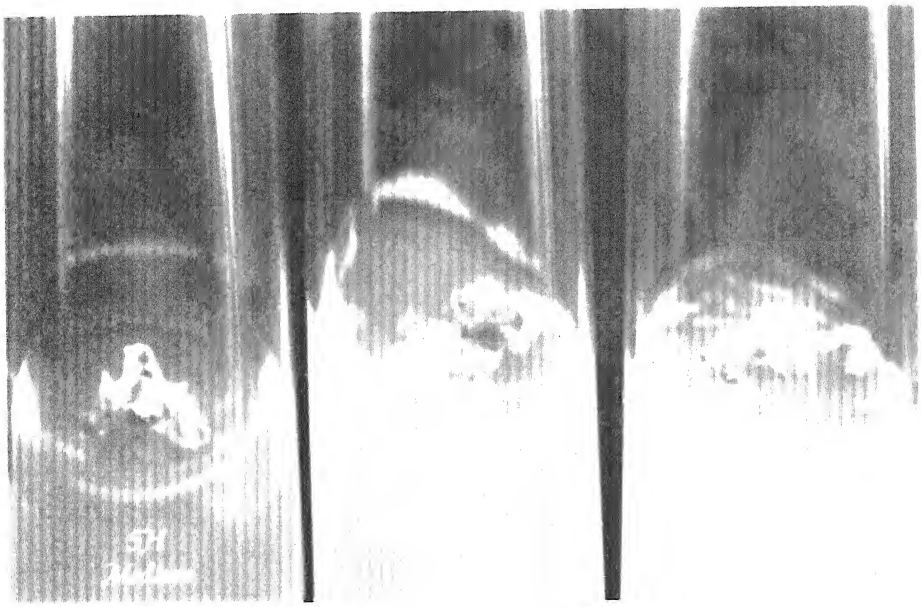


Fig.2 - Epicotyl Explant

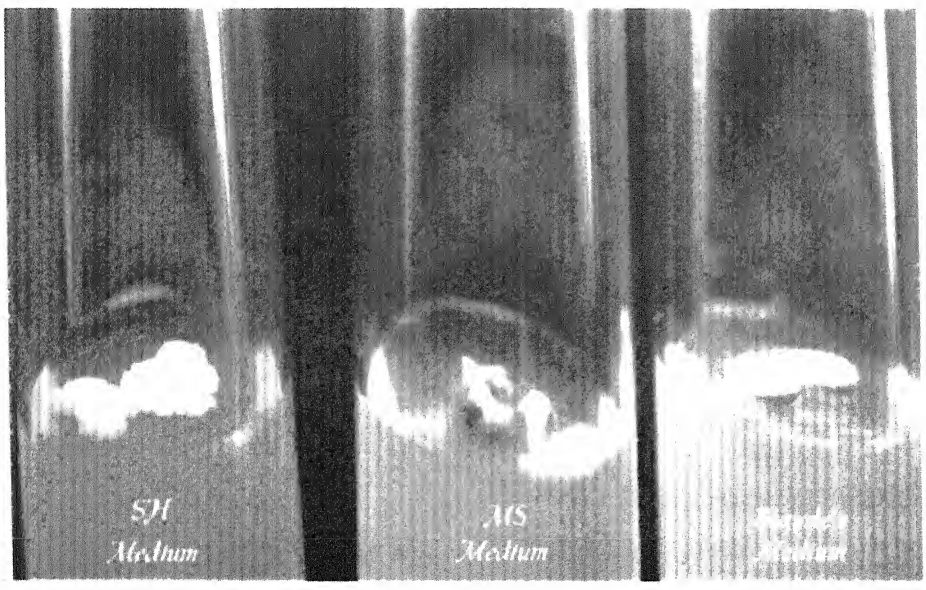


Fig.3 - Cotyledon Explant

Plate - 2

Callus Induction

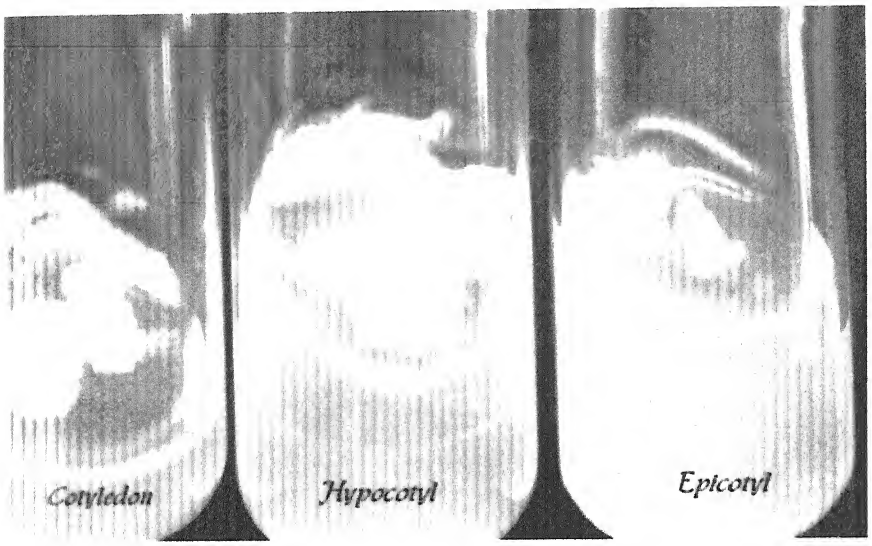


Fig. 1 - MS Medium

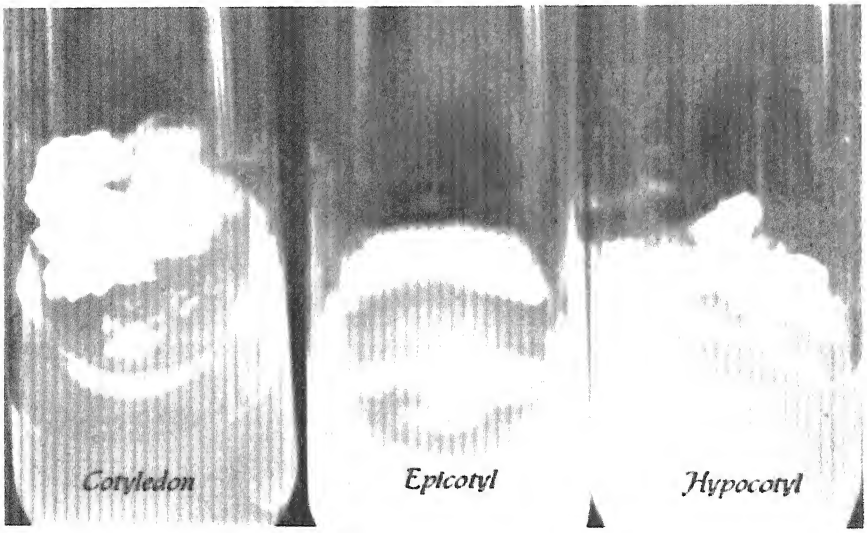


Fig.2 - SH Medium

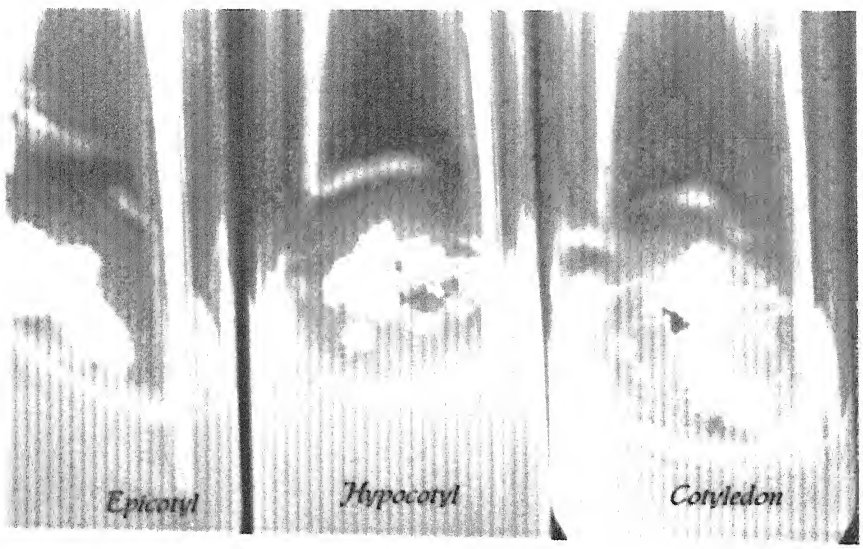


Fig.3 - Blaydo's Medium

4.1.2. Callus induction -

Calli were induced in eight genotypes of lucerne from three explants *viz.*, hypocoty, epicotyl and cotyledon on various culture media. Callus response was recorded in terms of callus induction frequency (% explant responded) and callus quality (colour and texture) The calli exhibited a range of colour, such as white, whitish yellow or yellowish white, light yellow, yellow, green and brown in combination with different types of texture, such as vitrious, granular, friable and nodular (Plate-5a & 5b, Fig.1–5). Callus colour and texture were recorded on a visual score scale ranging from 1 to 4, separately.

4.1.2.1. Hypocotyl Explant

(a) Callus induction frequency

The callus induction response of hypocotyl explant of the eight genotypes was observed to be highly significant among the media, genotypes and their interactions (Table-4.3). The best average was seen in IG-1212 (98.3%) followed by IL-75 (91.4%). The other four genotypes, C-10 (86.7%), A-3 (88.6%), LLC-9 (83.7%), AL-95-12 (88.6%) were statistically at par. Minimum callus induction frequency was recorded in LLC-3 (76.0%) which was at par with Anand-2 (77.7%).

Among different media combinations tried, the highest callus induction frequency (99.5%) was recorded on MS medium with 2.0 mg/l 2,4-D, 1.0 mg/l NAA and 0.2 mg/l BAP (Plate-3, Fig.1-3). SH medium containing1.0 mg/l kinetin with 1.0 mg/l NAA (94.5%) and 2.0 mg/l NAA (93.0%) were the next to the best and SH medium with 2.0 mg/l 2,4-D, 1.0 mg/l NAA and 0.2 mg/l BAP (93.0%) also performed equally good (Plate-4, Fig.1). At higher concentration of NAA (4.0 mg/l), the callus induction response was reduced (89.0%) as compared to that in lower (1.0 and 2.0 mg/l) concentrations of NAA applied with SH medium. On SH medium with 1.0 mg/l kinetin, inclusion of different concentrations of IAA (2.0 and 4.0 mg/l)

Table 4.3 - Effect of genotype and media composition on callus induction frequency in hypocotyl explant

Media composition	Genotypes								
	C-10	IG-1212	Il-75	A-3	LLC-9	LLC-3	Anand-2	AL-95-12	Mean
SH+1.0 mg/l NAA+ 1.0 mg/l kinetin	100.0	100.0	88.0	88.0	100.0	100.0	92.0	96.0	94.5 ab
SH+2.0 mg/l NAA+1.0 mg/l kinetin	100.0	100.0	76.0	88.0		96.0	92.0	92.0	93.0 bc
SH+4.0 mg/l NAA+1.0 mg/l kinetin	88.0	88.0	100.0	100.0	88.0	60.0	92.0	96.0	89.0 bcd
MS+2.0 mg/l 2,4-D+ 1.0 mg/l NAA+0.2 mg/l BAP	100.0	100.0	96.0	100.0	100.0	100.0	100.0	100.0	99.5 a
SH+2.0 mg/l 2,4-D+ 1.0 mg/l NAA+0.2 mg/l BAP	100.0	100.0	100.0	100.0	80.0	64.0	100.0	100.0	93.0 bc
SH+2.0 mg/l IAA+ 1.0 mg/l kinetin	60.0	100.0	96.0	72.0	68.0	60.0	36.0	64.0	69.5 e
SH+4.0 mg/l IAA+1.0 mg/l kinetin	60.0	100.0	92.0	72.0	50.0	52.0	32.0	72.0	66.3 e
Mean	86.7 bcd	98.3 a	91.4 b	88.6 bc	83.7 cde	76.0 f	77.7 f	88.6 bc	

Test - F value
Genotype - 11.49**
Media - 42.71**
Interaction - 5.42**

resulted in poor callus induction (69.5% and 66.3%, respectively) as compared to other auxins.

Lower concentration of NAA (1.0 mg/l) with 1.0 mg/l kinetin was better responded in LLC-3 (100.0%), AL-95-12 (96.0%) and IL-75 (88.0%) as compared to 2.0 mg/l concentration of NAA. On SH medium, C-10, IG-1212, A-3 and LLC-9 were statistically at par when supplemented in 1.0 and 2.0 mg/l concentrations of NAA. However at 4.0 mg/l concentration of NAA, only IL-75 (100.0 %) and A-3 (100.0 %) responded better than 1.0 and 2.0 mg/l concentrations of NAA. Thus, with the increase in NAA concentrations, the callus induction response decreased in C-10, IG-1212, LLC-9 and LLC-3. Such significant decreases were not observed in IL-75, A-3 and AL-95-12. Callus induction response in Anand-2 (92.0%) was at par in all the concentrations of NAA.

With the same combination of growth regulators (2.0 mg/l 2,4-D, 1.0 mg/l NAA and 0.2 mg/l BAP), MS medium was found better than SH for LLC-9 (100.0 %) and LLC-3 (100.0 %), while other genotypes such as C-10, IG-1212, A-3, Anand-2 and AL-95-12 were statistically at par for callus induction response on both SH and MS media. Conversely, in case of IL-75, SH medium was found better than MS. Significantly higher callus induction was observed in IL-75 (96.0%), LLC-9 (68.0%) LLC-3 (60.0 %) and Anand--2 (36.0%), at lower concentration of IAA (2.0 mg/l) as compared to its application in higher concentration of IAA (4.0 mg/l), where as C-10, IG-1212 and A-3, responded similarly at both the concentrations. At high concentration of IAA (4.0 mg/l) significant increase in callus induction response was found only in AL-95-12 (72.0%).

(b) Callus colour

Effect of different genotypes and media on callus colour from hypocotyl explant is presented in table 4.4. Genotypic and media differences for colour of hypocotyl derived callus were significant. The genotype IG-1212 scored maximum (3.21) for callus colour. This score indicates that most of the callus induced in this genotype was light yellow and greenish white. The least score (2.21) for colour was recorded

Table 4.4 - Effect of genotypes and media on colour of callus from hypocotyl explant

Genotypes	SH+1.0 mg/l NAA+ 1.0 mg/l kinetin	SH+2.0 mg/l NAA+1.0 mg/l kinetin	SH+4.0 mg/l NAA+1.0 mg/l kinetin	MS+2.0 mg/l 2,4-D+ 1.0 mg/l NAA+0.2 mg/l BAP	SH+2.0 mg/l 2,4-D+ 1.0 mg/l NAA+0.2 mg/l BAP	SH+2.0 mg/l IAA+ 1.0 mg/l kinetin	SH+4.0 mg/l IAA+1.0 mg/l kinetin	Mean
C-10	3.5	3.5	2.5	3.5	1.0	1.0	0.5	2.21 (b)
IG-1212	3.5	2.5	3.0	3.5	3.0	3.5	3.5	3.21 (a)
IL-75	3.5	3.5	3.0	3.5	3.5	1.0	1.0	2.71 (ab)
A-3	3.0	3.0	1.5	3.5	3.0	1.5	1.5	2.43 (ab)
LLC-9	2.0	3.5	2.5	3.5	3.5	2.0	2.0	2.71 (ab)
LLC-3	3.5	3.5	2.5	4.0	3.5	1.0	1.0	2.71 (ab)
Anand-2	3.0	3.0	3.5	3.5	3.5	1.0	1.0	2.64 (ab)
AL-95-12	2.0	2.0	4.0	3.5	4.0	1.0	1.0	2.5 (a)
Mean	3.0 [b]	3.06 [ab]	2.81 [b]	3.56 [a]	3.13 [ab]	1.5 [c]	1.44 [c]	-

Mean value with different alphabets in a row [] and column () are significantly different as per Kruskal-Wallis non parameteric statistics.

by genotype C-10. This score indicates for deep and light yellow callus. All other genotypes were comparable to both of these genotypes. The MS medium with 2.0 mg/l 2,4-D, 1.0 mg/l NAA and 0.2 mg/l BAP scored (3.56) for callus colour and least score (1.44) was exhibited by SH medium with 4.0 mg/l IAA and 1.0 mg/l kinetin. This least score indicates for brown and deep yellow callus on this medium. This overall trend of callus colour response on different media was also observed at individual genotype level.

(c) Callus texture

Effect of different genotypes and media on texture of callus from hypocotyl explant is given in table 4.5. Significant differences for hypocotyls derived callus were observed in different media and genotypes. Best callus texture was recorded in genotype Anand-2 (2.14) and AL-95-12 (2.14) which indicated that most of the callus was granular with little bit of friable callus. Poor (watery and granular) texture of callus was recorded in genotype LLC-9 (1.57) which was at par with A-3 (1.71) and LLC-9 (1.57). Maximum score for callus texture was recorded in SH medium with 2.0 mg/l 2,4-D, 1.0 mg/l NAA and 0.2 mg/l BAP (2.13), which was comparable to SH medium with 1.0 mg/l kinetin and 1.0 mg/l NAA or 2.0 mg/l NAA and MS medium with 2.0 mg/l 2,4-D, 1.0 mg/l NAA and 0.2 mg/l BAP. SH medium with 4.0 mg/l NAA and 1.0 mg/l kinetin exhibited the least callus texture score that was comparable to that of SH medium with 1.0 mg/l kinetin and 2.0 mg/l IAA or 4.0 mg/l IAA

4.1.2.2. Epicotyl Explant

(a) Callus induction frequency

Highly significant differences were observed among genotypes, media and their interactions with regards to callus induction frequency from epicotyl explants (Table-4.6). Among all the eight genotypes, the maximum response was observed in LLC-9 (59.6 %) followed by Anand -2 (57.6%), C-10 (56.4%) and LLC-3 (55.6 %) which were statistically at par. However, genotype A-3 recorded the lowest

Table 4.5 - Effect of genotypes and media on texture of callus from hypocotyl explant

Genotypes	SH+1.0 mg/l NAA+ 1.0 mg/l kinetin	SH+2.0 mg/l NAA+1.0 mg/l kinetin	SH+4.0 mg/l NAA+1.0 mg/l kinetin	MS+2.0 mg/l 2,4-D+ 1.0 mg/l NAA+0.2 mg/l BAP	SH+2.0 mg/l 2,4-D+ 1.0 mg/l NAA+0.2 mg/l BAP	SH+2.0 mg/l IAA+ 1.0 mg/l kinetin	SH+4.0 mg/l IAA+1.0 mg/l kinetin	Mean
C-10	2	2	2	2	2	2	2	2.0 (a)
IG-1212	2	2	2	2	2	2	2	2.0 (a)
IL-75	2	2	2	2	2	2	2	2.0 (a)
A-3	2	2	1	2	1	2	2	1.71 (ab)
LLC-9	2	2	1	2	2	1	1	1.57 (b)
LLC-3	2	2	1	2	2	2	2	1.86 (ab)
Anand-2	2	2	2	2	3	2	2	2.14 (a)
AL-95-12	2	2	2	2	3	2	2	2.14 (a)
Mean	2 [ab]	2 [ab]	1.63 [c]	2 [ab]	2.13 [a]	1.88 [bc]	1.88 [bc]	-

Mean value with different alphabets in a row [] and column () are significantly different as per Kruskal-Wallis non parameteric statistics.

Table 4.6 - Effect of genotype and media composition on callus induction frequency (%) in epicotyl explant of lucerne.

Media composition	Genotypes								
	C-10	IG-1212	IL-75	A-3	LLC-9	LLC-3	Anand-2	AL-95-12	Mean
SH+1.0 mg/l NAA +1.0 mg/l kinetin	28.0	26.0	28.0	14.0	60.0	46.0	52.0	44.0	37.3 d
SH+2.0 mg/l NAA +1.0 mg/l kinetin	40.0	34.0	36.0	44.0	52.0	46.0	52.0	36.0	42.5 d
SH+4.0 mg/l NAA+1.0 mg/l kinetin	66.0	28.0	56.0	46.0	62.0	52.0	60.0	54.0	53.0 c
MS+2.0 mg/l 2, 4-D+ 1.0 mg/l NAA+0.2 mg/l BAP	68.0	56.0	52.0	44.0	68.0	74.0	62.0	60.0	60.5 ab
SH+2.0 mg/l 2, 4-D+1.0 mg/l NAA+0.2 mg/l BAP	80.0	78.0	52.0	52.0	56.0	60.0	62.0	64.0	63.0 a
SH+2.0 mg/l IAA+ 1.0 mg/l kinetin	-	-	-	-	-	-	-	-	-
SH+4.0 mg/l IAA+1.0 mg/l kinetin	-	-	-	-	-	-	-	-	-
Mean	56.4 abc	44.4 ef	44.8 ef	40.0 f	59.6 a	55.6 abcd	57.6 ab	51.6 bcde	-

Test	-F value
Genotype	- 7.83**
Media	- 29.54**
Interaction	- 2.81**

callus induction frequency (40.0 %), which was at par with IG-1212 (44.4%) and IL-75 (44.8%).

Among different media combinations, the highest callus induction frequency was recorded on SH medium with 2.0 mg/l 2,4-D, 1.0 mg/l NAA and 0.2 mg/l BAP (63.0%) which was at par with MS medium containing the same growth regulators (60.5%). On SH medium with 1.0 mg/l kinetin, inclusion of lower concentrations (1.0 – 2.0 mg/l) of NAA resulted in poor callus induction (37.3%-42.5%), where as, at a high concentration of NAA (4.0 mg/l), the response was comparatively better (53.0%).

With the increase in NAA concentration, the callus induction response improved considerably in case of genotypes C-10, IL-75, A-3, LLC-3 and Anand-2, while such response was not noticed in case of IG-1212, LLC-9 and AL-95-12. Incorporation of 2,4-D @ 2.0 mg/l together with 1.0 mg/l NAA significantly increased callus induction in all the genotypes except in LLC-9. With the same growth regulator combinations (2.0 mg/l 2,4-D, 1.0 mg/l NAA and 0.2 mg/l BAP), SH medium was found better than MS for C-10 (80.0%), IG-1212 (78.0 %), A-3 (52.0%) and AL-95-12 (64.0%), while both the media with these growth regulator combinations were at par in case of IL-75 (52.0%) and Anand-2 (62.0%) for callus induction response. In case of LLC-9 and LLC-3, MS medium was superior to SH medium exhibiting maximum callus induction upto 68.0 % and 74.0 %, respectively.

(b) Callus colour

The effect of different genotypes and media on colour of callus from epicotyl explant are shown in table 4.7. In epicotyl explant, significant differences for callus colour were observed in different genotypes and media. All the genotypes and media recorded the visual score of more than 3 for callus colour. This suggested that most of the callus induced from this explant was light yellow, greenish and greenish white. Genotype IG-1212 (3.8) and SH medium with 2.0 mg/l 2,4-D, 1.0 mg/l NAA and 0.2 mg/l BAP (3.75) recorded the maximum score for callus colour.

Table 4.7 - Effect of genotypes and media on colour of callus from epicotyl explant

Genotypes	SH+1.0 mg/l NAA+ 1.0 mg/l kinetin	SH+2.0 mg/l NAA+1.0 mg/l kinetin	SH+4.0 mg/l NAA+1.0 mg/l kinetin	MS+2.0 mg/l 2,4-D+ 1.0 mg/l NAA+0.2 mg/l BAP	SH+2.0 mg/l 2,4-D+ 1.0 mg/l NAA+0.2 mg/l BAP	SH+2.0 mg/l IAA+ 1.0 mg/l kinetin	SH+4.0 mg/l IAA+1.0 mg/l kinetin	Mean
C-10	3.0	3.0	4.0	3.0	4.0	-	-	3.4 (ab)
IG-1212	3.5	4.0	4.0	3.5	4.0	-	-	3.8 (a)
IL-75	4.0	3.0	4.0	3.0	4.0	-	-	3.6 (ab)
A-3	3.0	3.0	4.0	3.5	4.0	-	-	3.5 (abc)
LLC-9	3.5	3.5	3.5	3.5	3.5	-	-	3.5 (abc)
LLC-3	3.0	3.0	3.0	3.5	3.0	-	-	3.1 (c)
Anand-2	3.0	3.0	3.0	4.0	4.0	-	-	3.4 (bc)
AL-95-12	3.5	3.5	3.5	3.5	3.5	-	-	3.5 (bc)
Mean	3.31 [bc]	3.25 [c]	3.62 [ab]	3.5 [abc]	3.75 [a]	-	-	-

Mean value with different alphabets in a row [] and column () are significantly different as per Kruskal-Wallis non parameteric statistics.

Least score for callus colour was recorded in genotype LLC-3 (3.1) and SH medium with 2.0 mg/l NAA and 1.0 mg/l kinetin (3.25).

(c) Callus texture

Effect of different genotypes and media on texture of callus from epicotyl explant is given in table 4.8. While genotypic differences for callus texture were found to be significant, the media differences for the same were non-significant. Except for the genotype C-10 and IL-75 which recorded the maximum score (2.0) for callus texture all other genotypes recorded 1.0 score for this trait.

4.1.2.3. Cotyledon Explant

(a) Callus induction frequency

The data on callus induction response of different genotypes on different media compositions are presented in Table-4.9. In cotyledon explants, highly significant differences were found in callus induction frequency among different media compositions and their interactions with genotypes. However, no significant differences were detected among the genotypes. Maximum callus induction frequency was recorded in IG-1212 (80.6%), followed by IL-75 (80.0%), LLC-9 (77.7%), LLC-3 (77.1%) and Anand-2 (77.1%). The genotypes, C-10 exhibited the lowest callus induction frequency (71.4%). However, all genotypes were at par statistically.

Among the different media compositions tried for callus induction frequency, the highest callus induction frequency (98.5 %) was recorded on MS medium containing 2.0 mg/l 2,4-D, 1.0 mg/l NAA and 0.2 mg/l BAP (Plate-3, Fig.1-3) which was at par with SH medium (95.5%) containing same growth regulators (Plate-4, Fig.2). On SH medium containing 1.0 mg/l kinetin, the callus induction frequency was statistically similar when supplemented with 1.0, 2.0 or 4.0 mg/l of NAA. Similarly, SH medium with 1.0 mg/l kinetin and low (2.0 mg/l) or high (4.0 mg/l) concentrations of IAA responded to callus induction to an equal extent.

Table 4.8 - Effect of genotypes and media on texture of callus from epicotyl explant

Genotypes	SH+1.0 mg/l NAA+ 1.0 mg/l kinetin	SH+2.0 mg/l NAA+1.0 mg/l kinetin	SH+4.0 mg/l NAA+1.0 mg/l kinetin	MS+2.0 mg/l 2,4-D+ 1.0 mg/l NAA+0.2 mg/l BAP	SH+2.0 mg/l 2,4-D+ 1.0 mg/l NAA+0.2 mg/l BAP	SH+2.0 mg/l IAA+ 1.0 mg/l kinetin	SH+4.0 mg/l IAA+1.0 mg/l kinetin	mean
C-10	2.0	2.0	2.0	2.0	2.0	-	-	2.0 (a)
IG-1212	2.0	1.0	1.0	1.0	1.0	-	-	1.2 (b)
IL-75	2.0	2.0	2.0	2.0	2.0	-	-	2.0 (a)
A-3	1.0	1.0	1.0	1.0	1.0	-	-	1.0 (b)
LLC-9	1.0	1.0	1.0	1.0	1.0	-	-	1.0 (b)
LLC-3	1.0	1.0	1.0	1.0	1.0	-	-	1.0 (b)
Anand-2	1.0	1.0	1.0	1.0	1.0	-	-	1.0 (b)
AL-95-12	1.0	1.0	1.0	1.0	1.0	-	-	1.0 (b)
Mean	1.37 [a]	1.25 [a]	1.25 [a]	1.25 [a]	1.25 [a]	-	-	-

Mean value with different alphabets in a row [] and column () are significantly different as per Kruskal-Wallis non parameteric statistics.

Table 4.9 - Effect on genotype and media composition on callus induction frequency (%) in cotyledon explant of lucerne

Media composition	Genotypes								
	C-10	IG-1212	IL-75	A-3	LLC-9	LLC-3	Anand-2	AL-95-12	Mean
SH+1.0 mg/l NAA+1.0 mg/l Kinetin	96.0	100.0	84.0	56.0	84.0	88.0	76.0	96.0	85.0 c
SH+2.0 mg/l NAA+1.0 mg/l kinetin	56.0	100.0	88.0	64.0	96.0	100.0	60.0	92.0	82.0 cde
SH+4.0 mg/l NAA+1.0 mg/l kinetin	48.0	76.0	100.0	100.0	88.0	88.0	88.0	80.0	83.5' cd
MS+2.0 mg/l 2,4-D+ 1.0 mg/l NAA+0.2 mg/l BAP	96.0	100.0	100.0	100.0	100.0	92.0	100.0	100.0	98.5 a
SH+2.0 mg/l 2,4-D+ 1.0 mg/l NAA+0.2 mg/l BAP	100.0	96.0	100.0	100.0	92.0	84.0	100.0	92.0	95.5 ab
SH+2.0 mg/l IAA+1.0 mg/l kinetin	64.0	44.0	52.0	40.0	48.0	40.0	56.0	36.0	47.5 f
SH+4.0 mg/l IAA+1.0 mg/l Kinetin	40.0	48.0	36.0	48.0	36.0	48.0	60.0	32.0	43.5 f
Mean	71.4	80.6	80.0	72.6	77.7	77.1	77.1	75.4	

Test - F value
Genotype - 0.95
Media - 50.49**
Interaction - 2.09**

It was found that the extent of callus induction in genotypes IL-75 and A-3 increased with the increasing concentration of NAA but in other genotypes such as C-10 and IG-1212, it decreased with increasing concentrations of NAA. Any of such trends was not observed in case of LLC-9, LLC-3 and Anand-2. At 4.0 mg/l NAA, IL-75 and A-3, both responded better than the other genotypes when the other media constituents remained the same.

(b) Callus colour

Effect of different genotypes and media on callus colour from cotyledon explant is given in table 4.10. Genotypic and media differences were significant with respect to callus colour from cotyledon explant. Genotype AL-95-12 (2.93) exhibited maximum score for callus colour that was dark yellow to light yellow followed by C-10 (2.79), IG-1212 (2.5), IL-75 (2.5), LLC-9 (2.29), LLC-3 (2.57) and Anand-2 (2.57) which were statistically at par with each other. Least score for callus colour was recorded in genotype A-3 (1.86) that was brown to dark yellow. MS medium with 2.0 mg/l 2,4-D, 1.0 mg/l NAA and 0.2 mg/l BAP recorded maximum score of callus colour. This score indicated for light yellow to greenish or whitish colour followed by SH medium (3.31) containing same growth regulators. SH medium with 1.0 mg/l kinetin and 2.0 mg/l IAA or 4.0 mg/l IAA recorded least callus colour (1.06) score which was indicated for brown colour of callus.

(c) Callus texture

Effect of the different genotypes and media on texture of the callus from cotyledon explant is presented in table 4.11. While genotypic differences for cotyledonary callus texture were significant, the media difference for the same were non significant. Except for the genotypes LLC-3 which recorded the least score for callus texture, all other genotypes were comparable for this trait having granular type of callus.

Table 4.10 - Effect of genotypes and media on colour of callus from cotyledon explant

Genotypes	SH+1.0 mg/l NAA+ 1.0 mg/l kinetin	SH+2.0 mg/l NAA+1.0 mg/l kinetin	SH+4.0 mg/l NAA+1.0 mg/l kinetin	MS+2.0 mg/l 2,4-D+ 1.0 mg/l NAA+0.2 mg/l BAP	SH+2.0 mg/l 2,4-D+ 1.0 mg/l NAA+0.2 mg/l BAP	SH+2.0 mg/l IAA+ 1.0 mg/l kinetin	SH+4.0 mg/l IAA+1.0 mg/l kinetin	Mean
C-10	3.0	3.5	3.5	4.0	3.5	1.0	1.0	2.79 (ab)
IG-1212	2.0	3.5	3.0	3.5	3.5	1.0	1.0	2.5 (abc)
IL-75	3.0	3.0	3.0	3.5	3.0	1.0	1.0	2.5 (abc)
A-3	1.5	1.5	1.5	3.5	3.0	1.0	1.0	1.86 (c)
LLC-9	2.5	2.5	2.0	2.5	3.5	1.5	1.5	2.29 (ab)
LLC-3	3.5	3.5	2.0	3.5	3.5	1.0	1.0	2.57 (ab)
Anand-2	3.0	3.5	3.0	3.5	3.0	1.0	1.0	2.57 (ab)
AL-95-12	3.5	3.5	4.0	4.0	3.5	1.0	1.0	2.93 (a)
Mean	2.75 [b]	3.06 [ab]	2.75 [b]	3.5 [a]	3.31 [ab]	1.06 [c]	1.06 [c]	-

Mean value with different alphabets in a row [] and column () are significantly different as per Kruskal-Wallis non parameteric statistics.

Table 4.11 - Effect of genotypes and media on texture of callus from cotyledon explant

Genotypes	SH+1.0 mg/l NAA+ 1.0 mg/l kinetin	SH+2.0 mg/l NAA+1.0 mg/l kinetin	SH+4.0 mg/l NAA+1.0 mg/l kinetin	MS+2.0 mg/l 2,4-D+ 1.0 mg/l NAA+0.2 mg/l BAP	SH+2.0 mg/l 2,4-D+ 1.0 mg/l NAA+0.2 mg/l BAP	SH+2.0 mg/l IAA+ 1.0 mg/l kinetin	SH+4.0 mg/l IAA+1.0 mg/l kinetin	Mean
C-10	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0 (ab)
IG-1212	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0 (a)
IL-75	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0 (a)
A-3	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0 (a)
LLC-9	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0 (ab)
LLC-3	2.0	2.0	1.0	2.0	2.0	1.0	1.0	1.57 (b)
Anand-2	2.0	2.0	3.0	2.0	1.0	2.0	2.0	2.0 (a)
AL-95-12	2.0	2.0	1.0	2.0	2.0	2.0	2.0	1.88 (ab)
Mean	2.0 [a]	2.0 [a]	1.88 [a]	2.0 [a]	1.88 [a]	1.88 [a]	1.88 [a]	-

Mean value with different alphabets in a row [] and column () are significantly different as per Kruskal-Wallis non parameteric statistics.

Plate - 3

Callus Induction on MS medium with 2.4-D

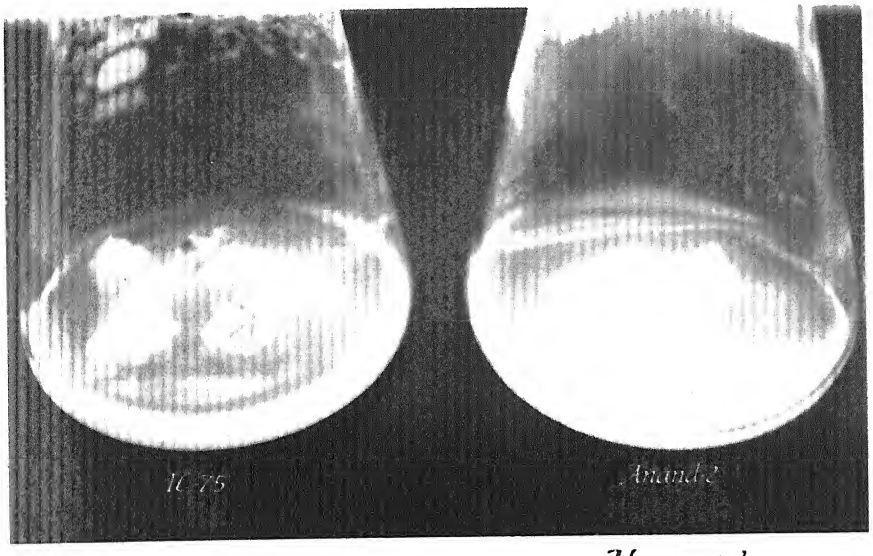


Fig.1 - Cotyledon Hypocotyl

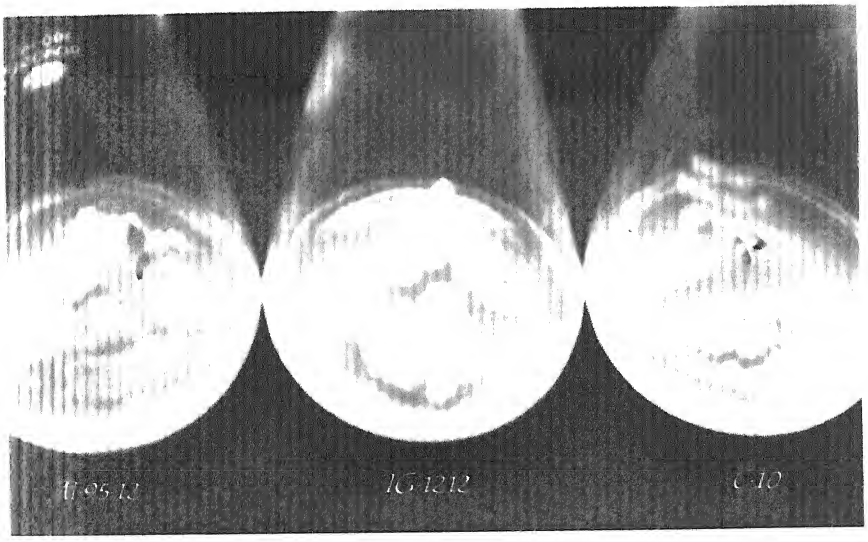


Fig.2 - Hypocotyl Cotyledon Hypocotyl

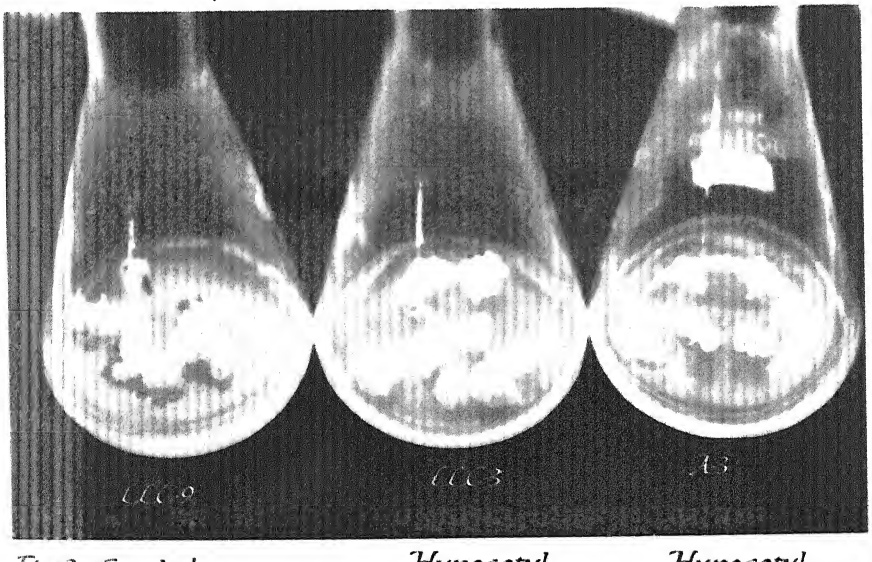


Fig.3 - Cotyledon Hypocotyl Hypocotyl

Plate - 4

Callus Induction on SH Medium with 2.4-D

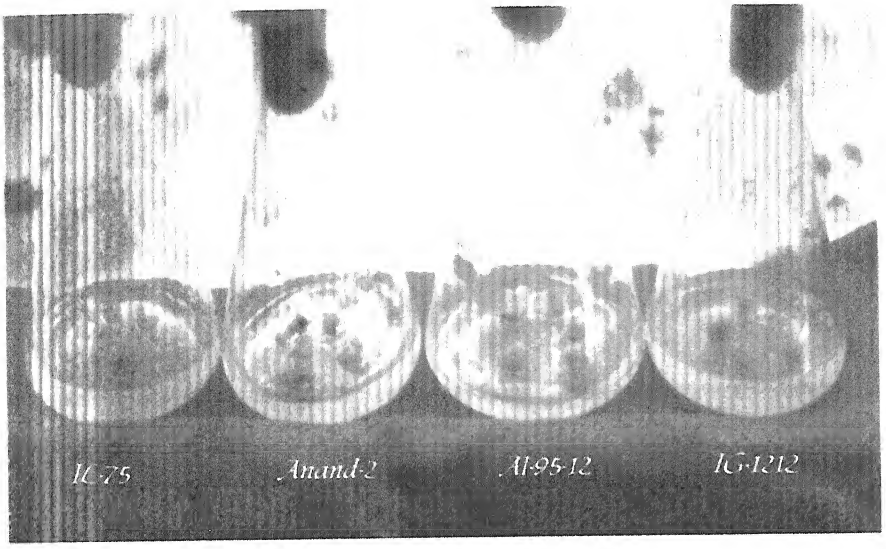


Fig.1 - Hypocotyl Explant

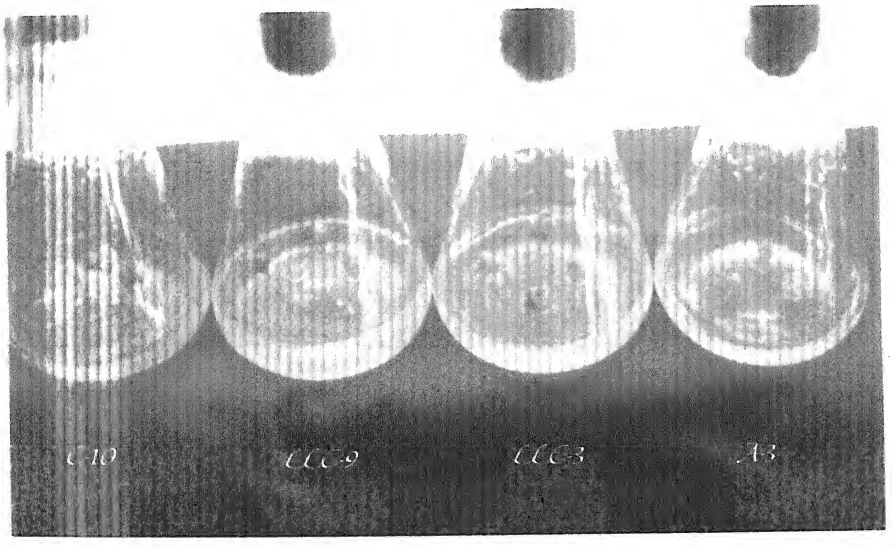


Fig.2 - Cotyledon Explant

Plate - 5a

Callus Quality

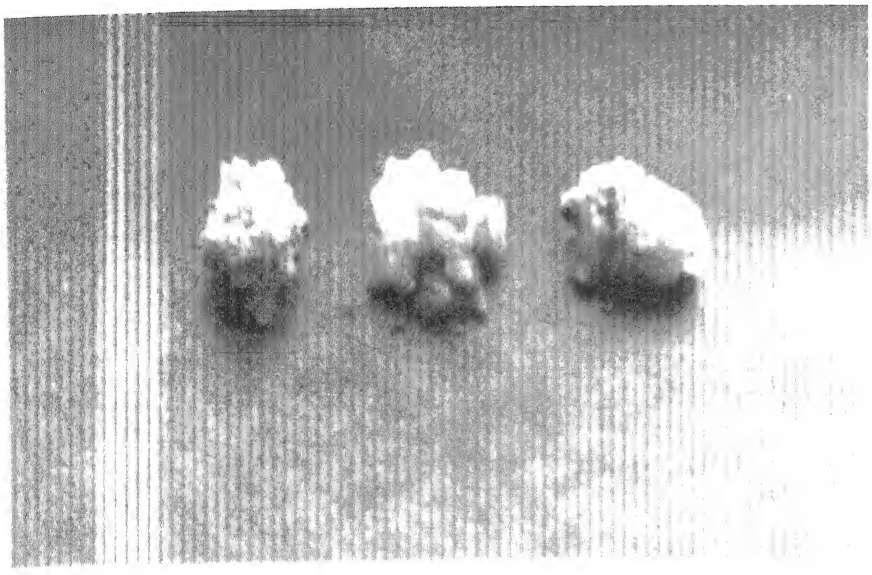


Fig.1 - Brown and Granular callus

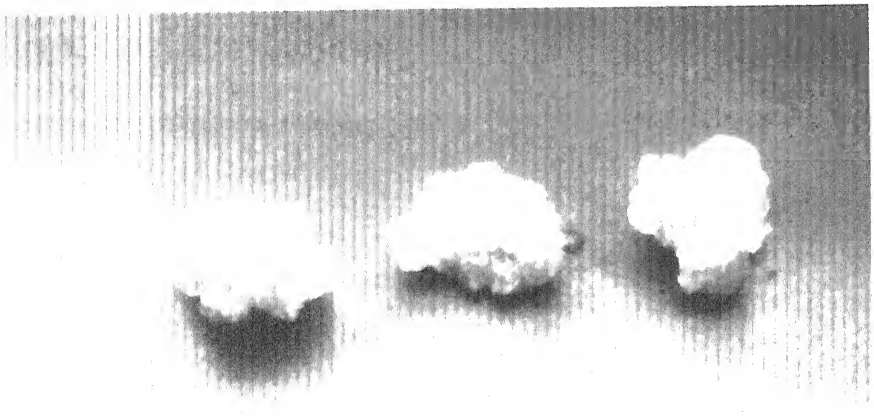


Fig.2 - Yellow, Granular and Friable callus

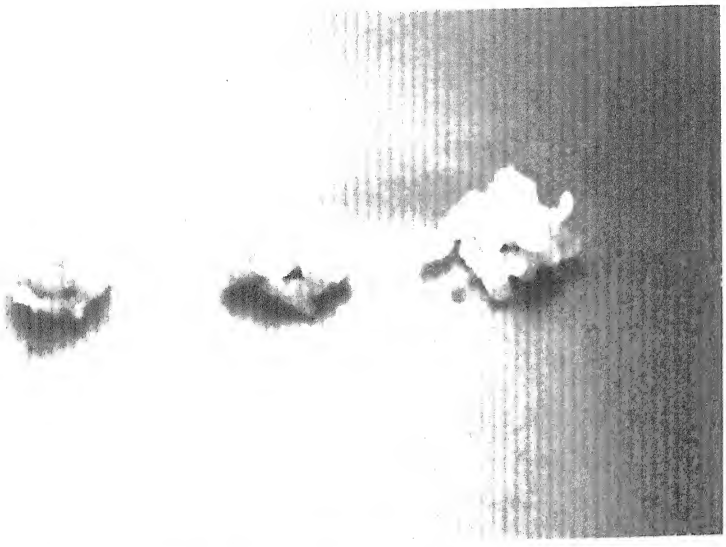


Fig.3 - Light yellow, Granular, Friable and Nodular callus

Plate - 5b

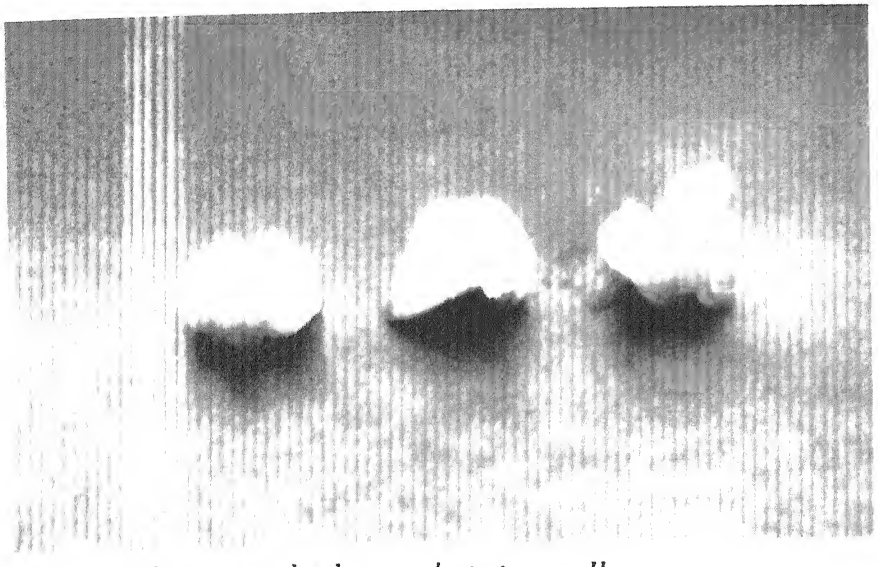


Fig.4 - Light yellowish white and vitrious callus

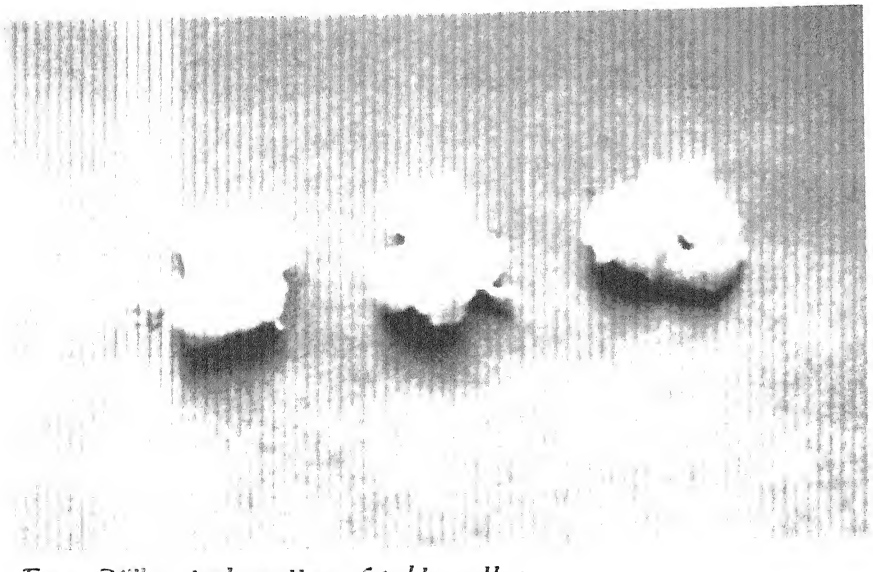


Fig.5 - White-light yellow, friable callus

4.1.3. Callus growth rate

The calli were initially raised on MS medium from the three explants and the luxuriantly growing calli from these explants were individually selected for callus growth rate experiments. A measured amount of callus in each case was taken for successive subcultures at a regular interval of 20-21 days on MS medium supplemented with 2.0 mg/l 2,4-D, 1.0 mg/l NAA and 0.2 mg/l BAP and maintained upto eighty days. Callus growth rate interms of fresh weight and dry weight of callus were measured in each case upto eighty days of calli growth.

Hypocotyl explant

The rate of callus growth in eight genotypes (LLC-9, LLC-3, Anand-2, AL-95-12, C-10, IL-75, IG-1212 and A-3) of lucerne from hypocotyl explant (Plate-6, Fig.1&2) exhibited genotype dependent growth behavior over 80 days in culture as measured by the increase in fresh weight (fig.1) and dry weight (fig.2) of calli, calculated on the initial explant weight basis. The percent callus growth rate on log scale indicated that in all the genotypes growth increased with increase in duration of culture. However, in Anand-2 alone on fresh callus weight basis and in Anand-2 and AL-95-12 on the basis of dry weight of callus the growth stabilized after 60 days. The maximum growth was observed after 40 days in LLC-9 which was steady till 60th days and again increased up to 80 days. Regular increase in growth rate was observed in calli of the genotypes LLC-3, C-10, IL-75, IG-1212 and A-3 even up to 80 days on both fresh and dry weight basis. At the end of 80 days, all the genotypes except Anand-2 were showing growth rate on log phase pattern and Anand-2 had plateaued for the fresh weight of callus and Anand-2 and AL-95-12 plateaued for dry weight of callus.

Epicotyl explant

The graph based on log scale, indicated the rate of callus growth in all the eight genotypes (LLC-3, LLC-9, Anand-2, AL-95-12, C-10, IL-75, IG-1212 and A-3) of lucerne as measured by the increase on fresh weight basis upto 80 days in culture (fig.3). All the genotypes exhibited steady and regular growth rate in all the passages

Plate - 6

Callus Growth Rate (hypocotyl)

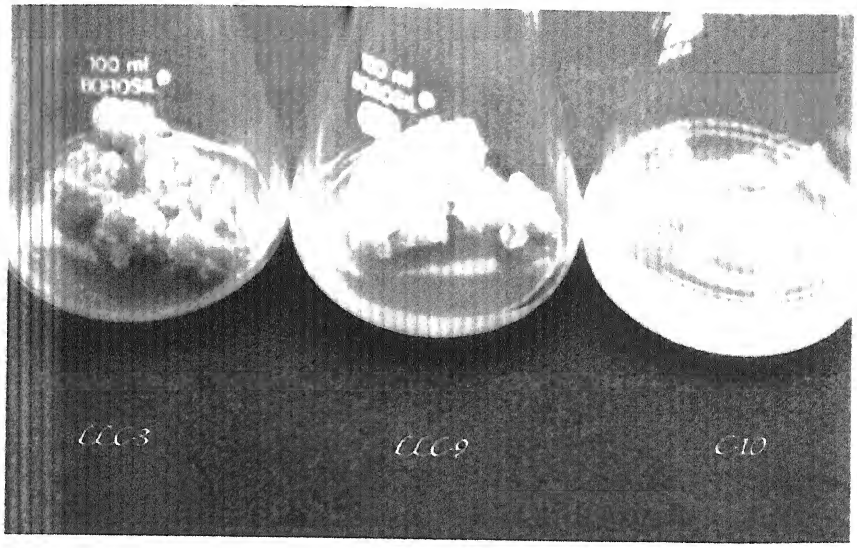


Fig.1

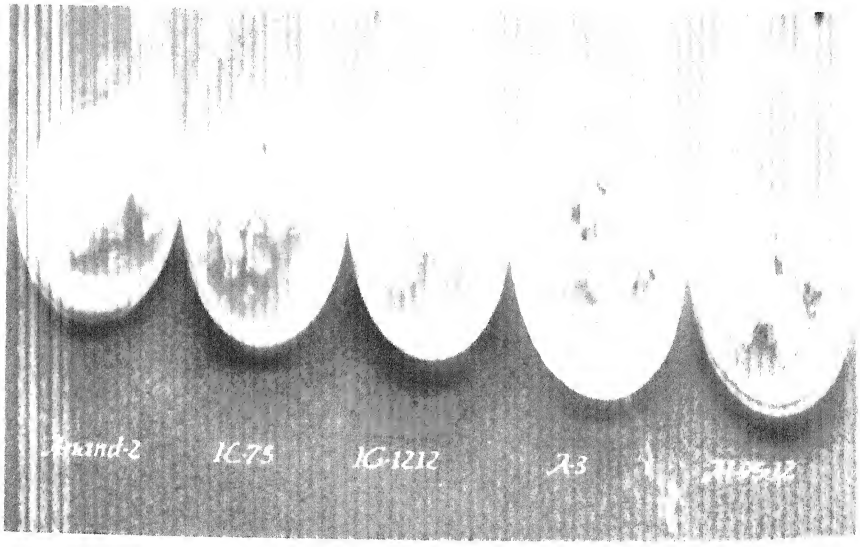


Fig.2

Fig. 1 - Growth rate of calli over the passeges as measured by increase in fresh weight of callus from hypocotyl explant of eight genotypes of lucerne

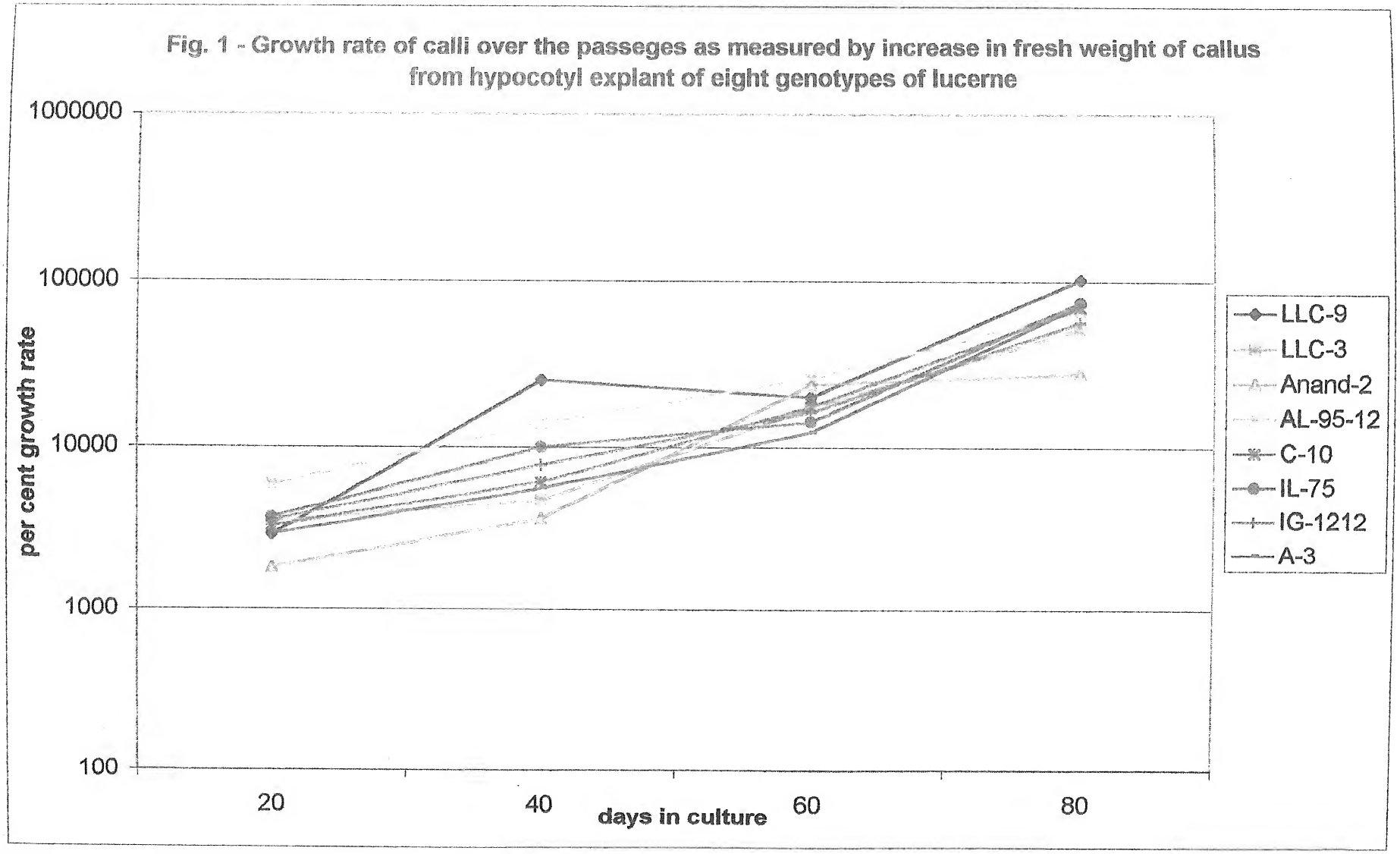


Fig.2 - Growth rate of calli over the passages as measured by increase in dry weight in hypocotyl explant of eight genotypes of lucerne

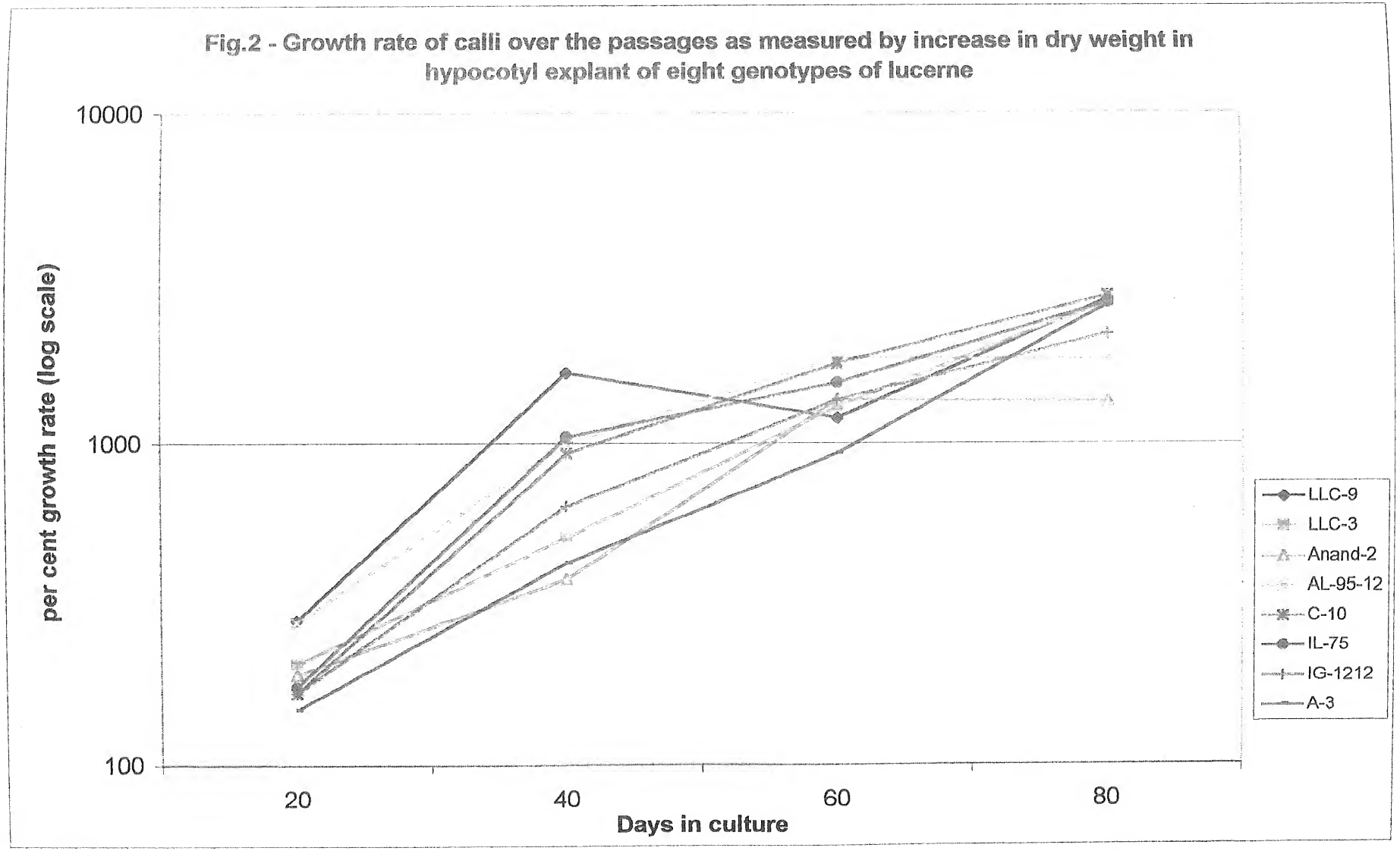
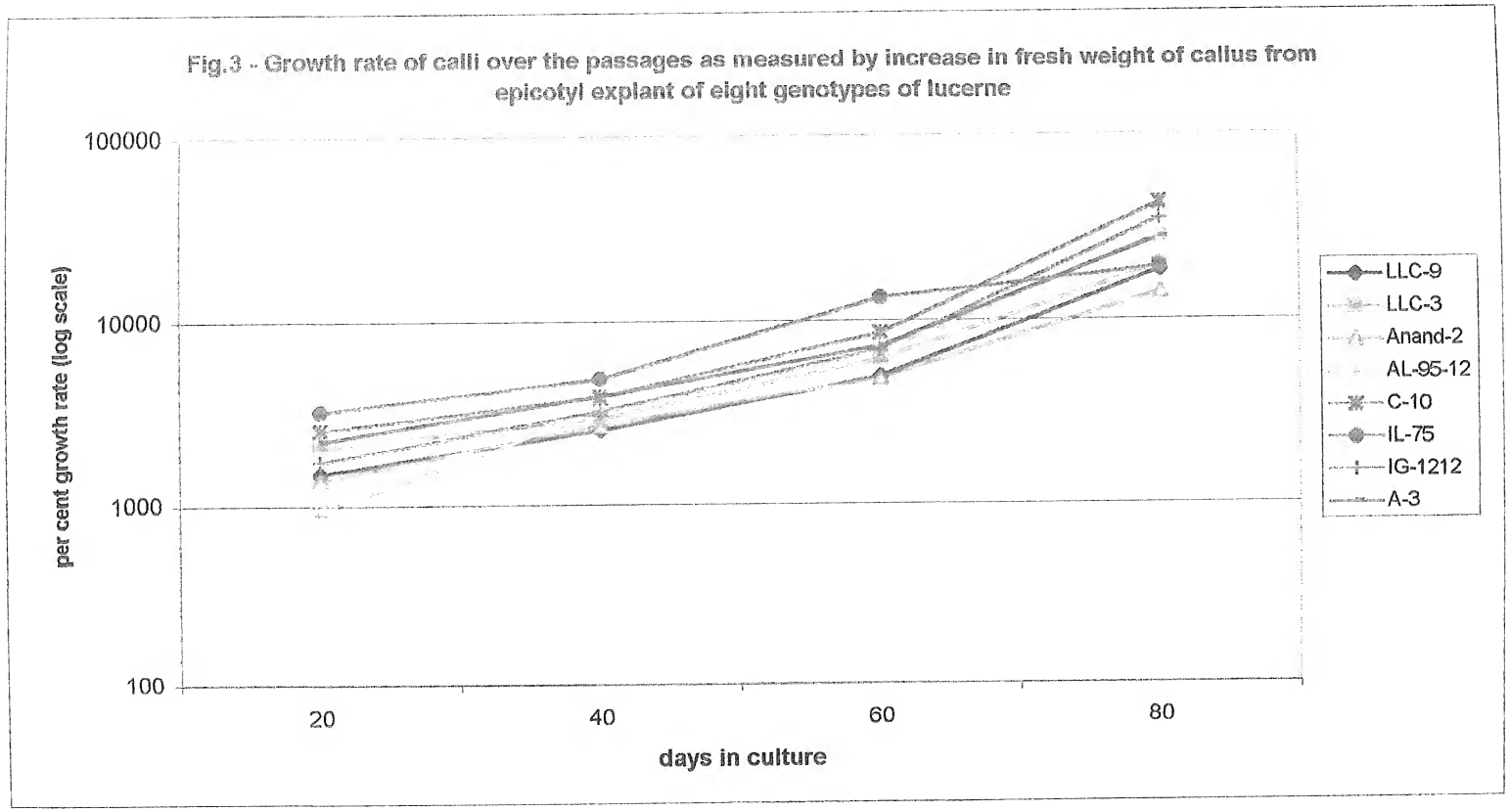


Fig.3 - Growth rate of calli over the passages as measured by increase in fresh weight of callus from epicotyl explant of eight genotypes of lucerne



except IL-75 in which the growth rate on the basis of fresh callus weight was nearly stabilized at 80 days in culture. The maximum callus growth rate at 40 and 60 days in culture was exhibited by IL-75 where as the maximum callus growth rate on fresh callus weight basis was found in the genotype C-10 observed at 80 days in culture. The minimum callus growth on the basis of fresh callus weight basis occurred in LLC-9 and Anand-2 genotypes both at 40 and 60 days in culture while it was minimum in Anand-2 at 80 days in culture.

The growth rate on the basis of dry weight of callus in all the eight genotypes is presented in figure-4. All the genotypes showed increase in the dry weight of callus upto 60 days in culture. The genotypes AL-95-12 LLC-3, C-10, IG-1212 and LLC-9 exhibited regular increase in dry weight of callus upto 80 days of culture where as the growth rate on the basis of dry weight of callus was stabilized between 60 and 80 days in culture in the genotypes IL-75, Anand-2 and A-3. The maximum dry weight of callus at 40 days was observed in IL-75. AL-95-12 performed best in terms of dry callus weight at 60 and 80 days in culture. The minimum callus dry weight at 40 days was observed in LLC-3 where as at 60 and 80 days cultures it was found in A-3 genotype.

Cotyledon explant

The graph, based on log scale, indicated the rate of callus growth in the eight genotypes of lucerne measured by the increase in fresh weight (fig.5) and increase and decrease in dry weight (fig.6) of the calli on the basis of initial explant weight. In the eight genotypes, callus growth rate increased with increasing days in culture on fresh weight basis but on basis of dry weight, it was observed that growth rate of callus increased up to 60 days and again the growth rate was decreased in LLC-9, AL-95-12 and IG-1212 up to 80 days, where as in LLC-3, IL-75, Anand-2, C-10, A-3 regular and steady growth was observed.

Fig.4 - Growth rate of calli over the passages as measured by increase in dry weight of callus from epicotyl explant of eight genotypes of lucerne

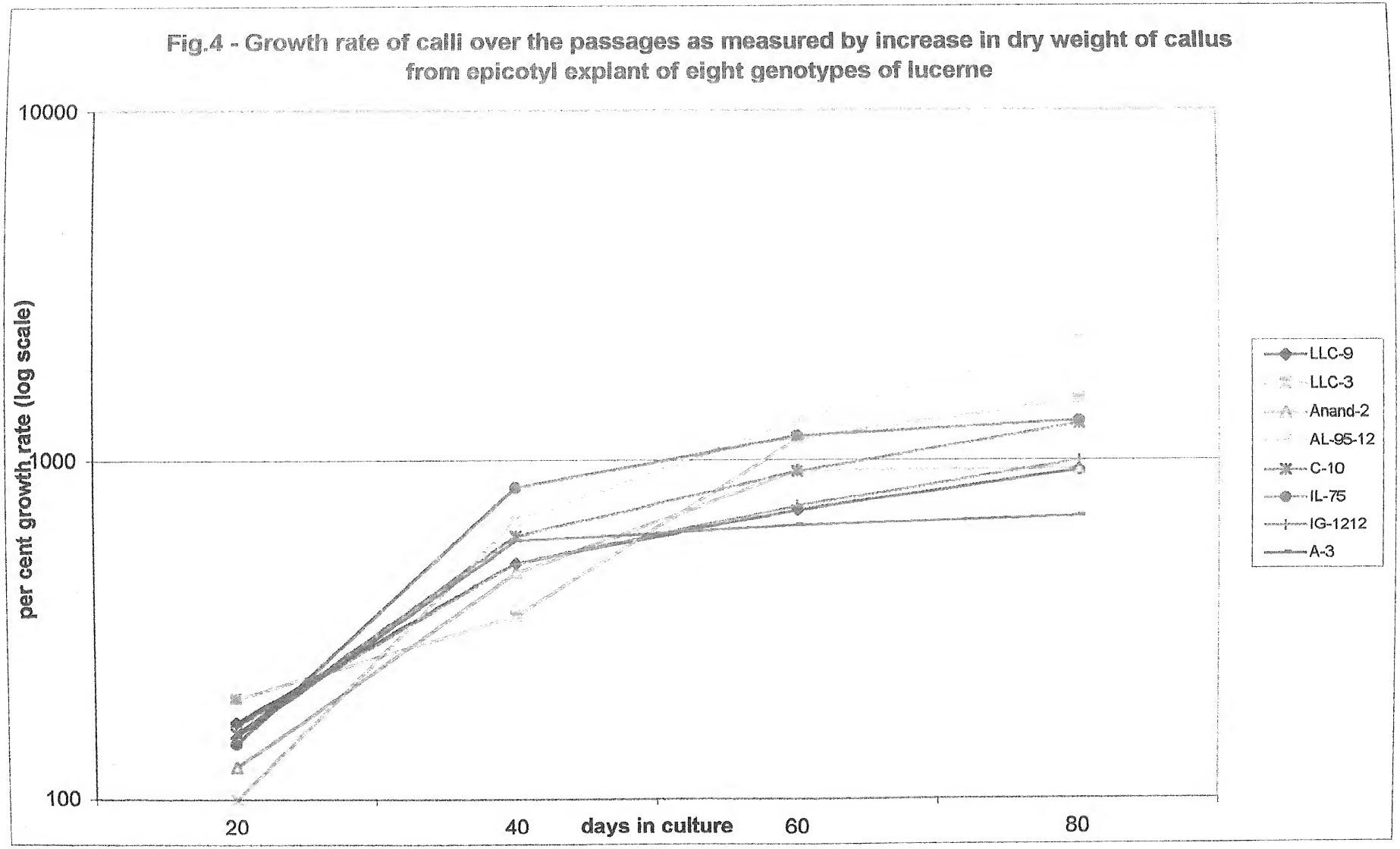


Fig.5 - Gowth rate of calli over the passages as measured by increase in fresh weight in cotyledon explant of eight gnotypes of lucerne

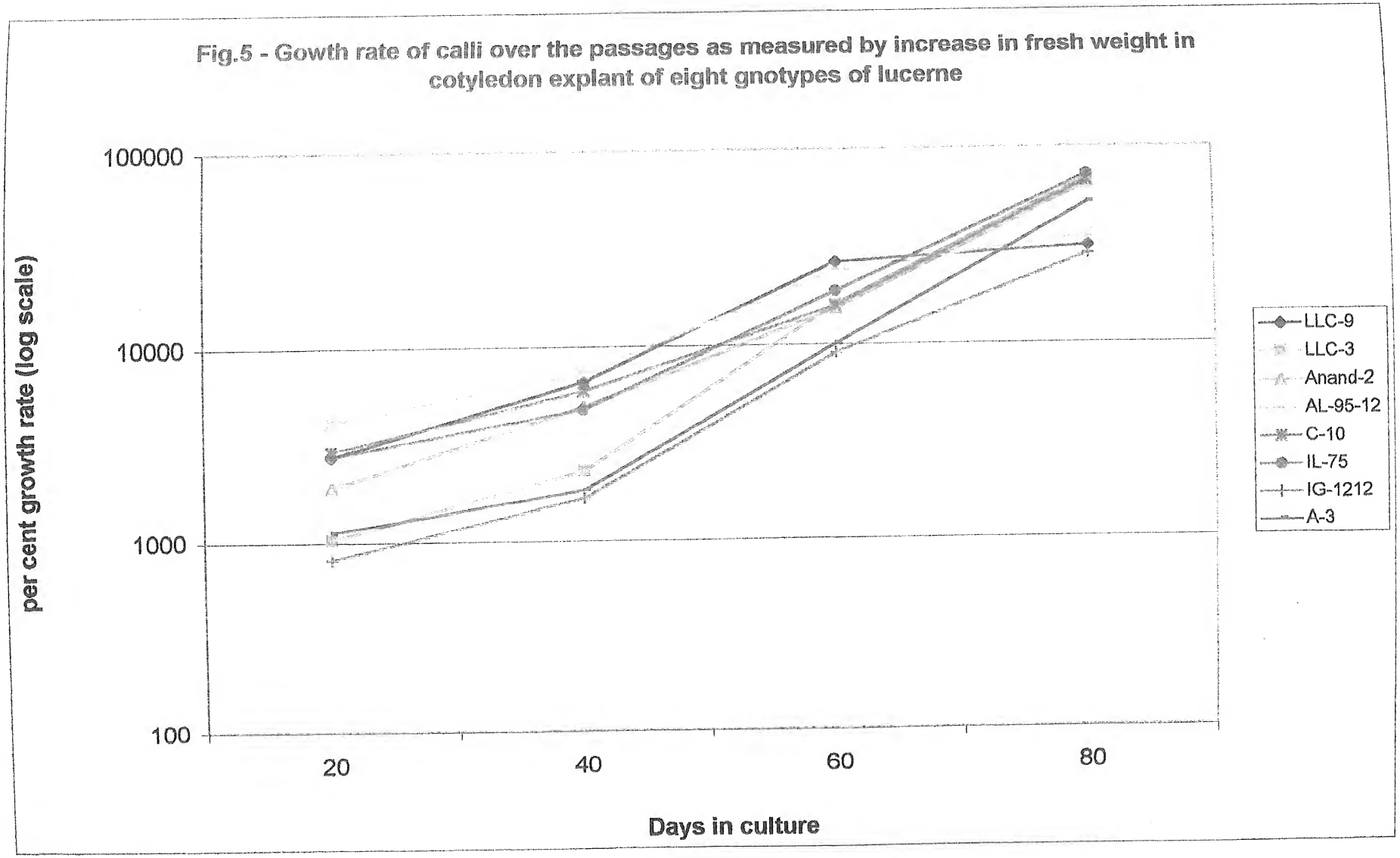
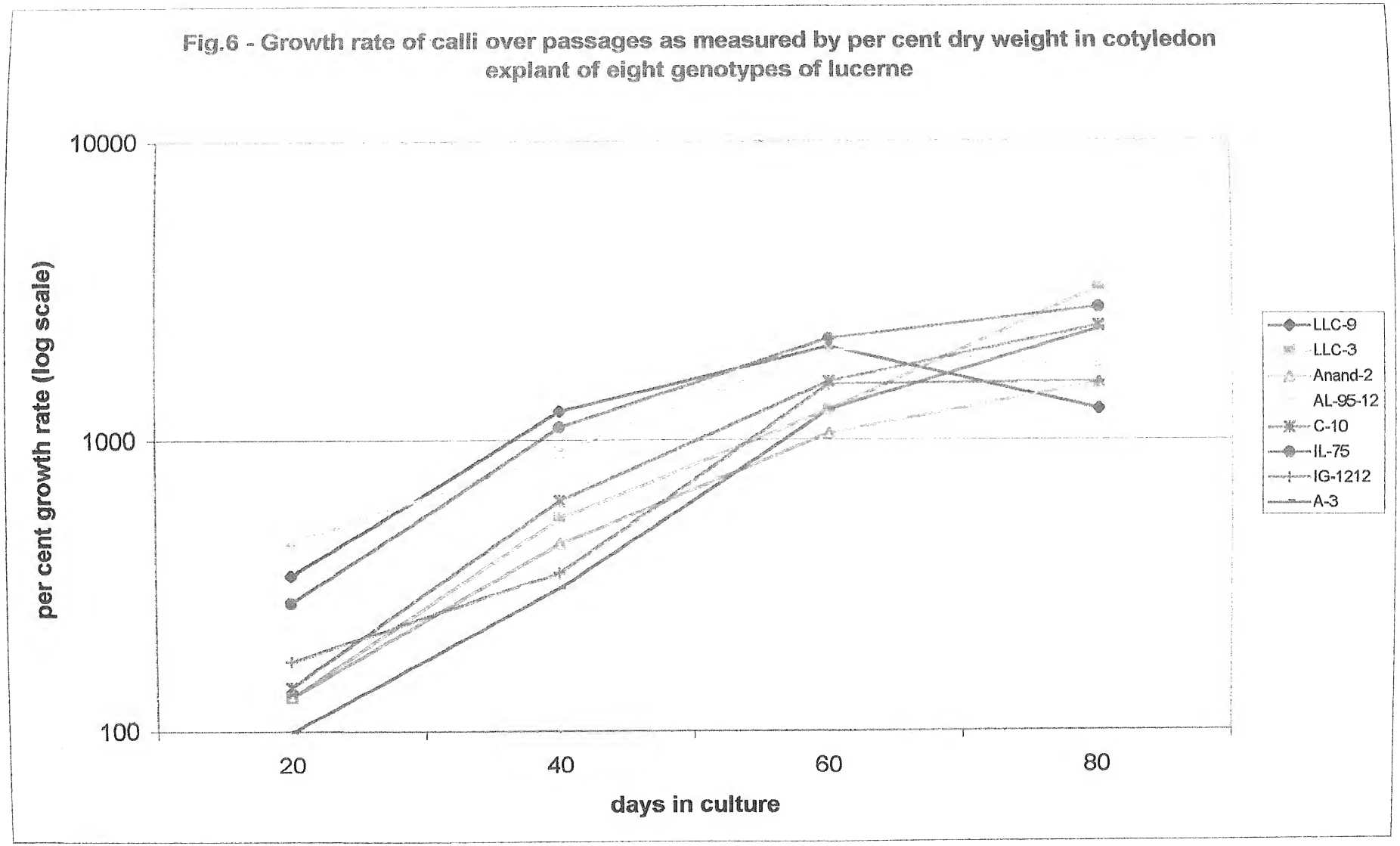


Fig.6 - Growth rate of calli over passages as measured by per cent dry weight in cotyledon explant of eight genotypes of lucerne



Dry matter content

The accumulation of dry matter content as calculated in terms of per cent dry matter of the calli during the subcultures at a regular interval of 20 days over the passages up to 80 days in the calli from different explants has been presented in figures 7-9.

Hypocotyl

Callus growth rate in hypocotyl as reflected by the initial increase in fresh weight content of the callus with days in culture indicated decreased accumulation of per cent dry matter content with increase in subculture duration, in general (fig.7). This trend was most evident in the genotypes LLC-9 and Anand-2, while in C-10, LLC-3, AL-95-12 and A-3, the dry matter content was more after 40 days in culture and decreased thereafter. In IL-75 and IG-1212 the dry matter content of callus was nearly stabilized between 40 and 60 days and thereafter it had a decreasing trend. The genotype C-10 had maximum dry matter content in the calli up to 40 days followed by Anand-2 and IL-75 which were almost similar. The maximum dry matter content at 60 days of culture was recorded in IL-75 followed by C-10 and IG-1212. The maximum dry matter content after 80 days of culture was recorded in LLC-3 and the minimum in AL-95-12 and LLC-9 genotypes.

Epicotyl

Accumulation of per cent dry matter content due to callus growth from epicotyl explant decrease with increase in duration in culture in all the eight genotypes with an initial increase between 20 and 40 days of callus growth in culture (fig.8). AL-95-12 had maximum dry matter content accumulated upto 40 days of culture followed by LLC-9.

The genotypes Anand-2 and AL-95-12 showed maximum dry matter accumulation in their calli at 60 days after culture followed by the genotype LLC-3. The maximum dry matter accumulation in the calli after 80 days of growth in culture was observed in the genotypes AL-95-12 and LLC-3. This was followed by IL-75 and

Fig.7 - Growth rate of calli as measured by per cent dry matter content over passages in callus from hypocotyl explant of eight genotypes of lucerne

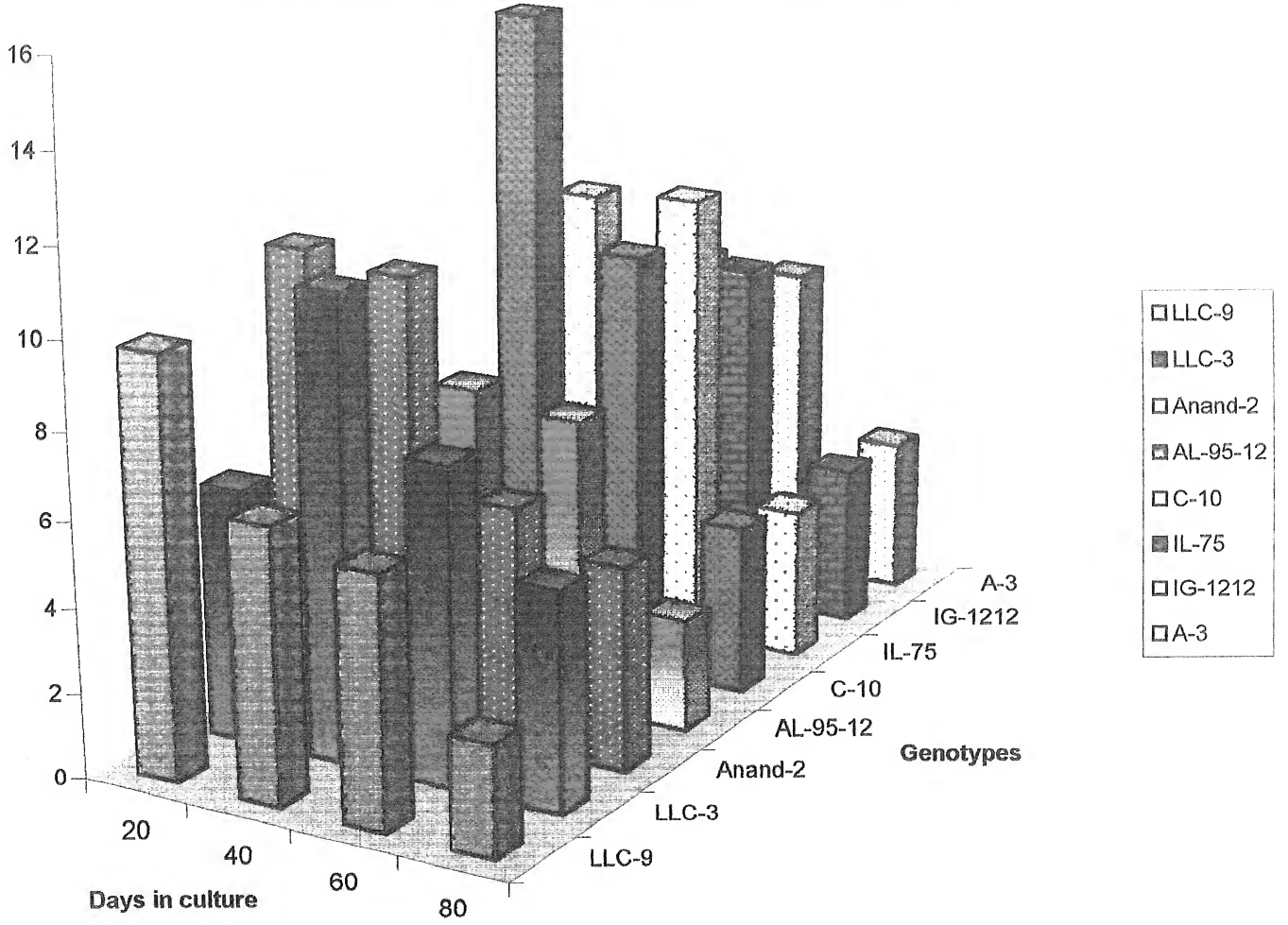
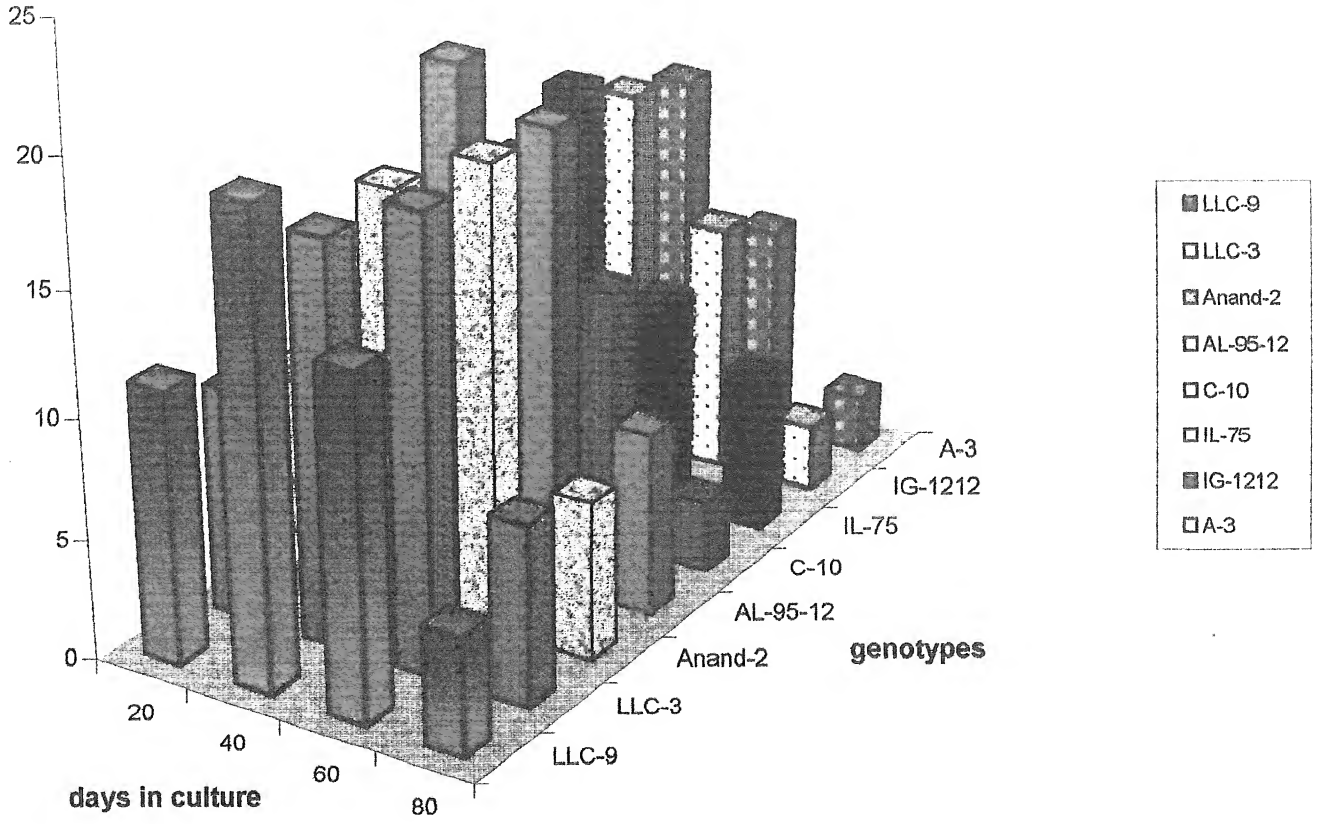


Fig.8 - Growth rate of calli as measured by per cent dry matter content over passages in callus from epicotyl explant of eight genotypes of lucerne



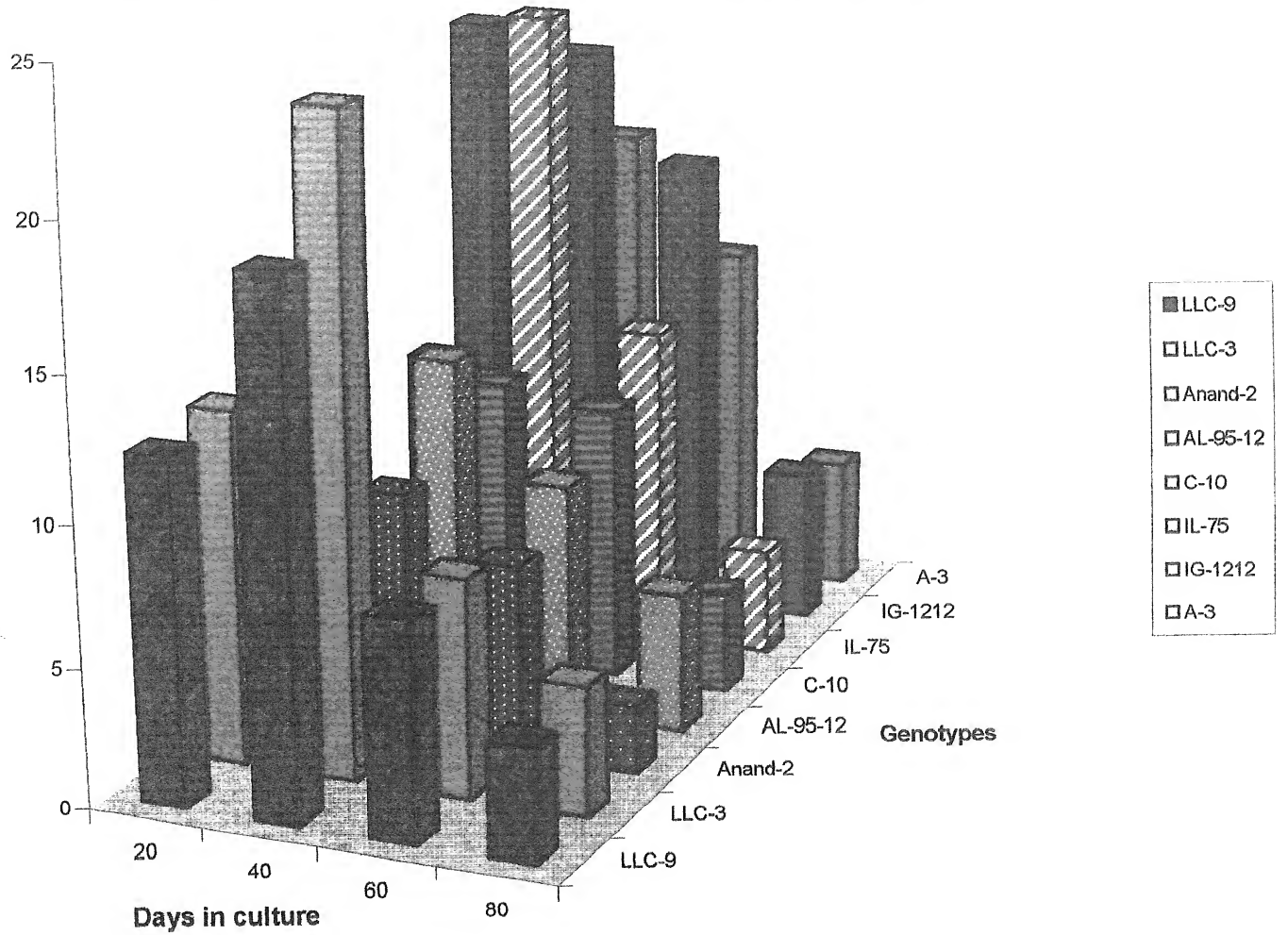
Anand-2. The minimum accumulation of dry matter content in the callus after 80 days of growth in culture was recorded in the genotype A-3. Genotype AL-95-12 showed maximum dry matter accumulation in the calli in general during entire period of callus growth of 80 days under study.

Cotyledon

Proportional accumulation of per cent dry matter content due to callus growth decreased with increase in days in culture from cotyledon explant in all the eight genotypes with an initial increase between 20 and 40 days of callus growth (fig.9). On comparing the growth rate increase among genotypes, IL-75 and LLC-3 had maximum dry matter accumulated up to 40 days after culture and then awards IG-1212 had maximum per cent dry matter accumulation rate 60 and 80 days after culture. While per cent dry matter content increased maximum upto 40 days in IL-75 and LLC-3, it was recorded minimum in Anand-2. The minimum per cent dry matter accumulation after 60 and 80 days after culture was also observed in Anand-2

The results of callus growth rate in terms of fresh callus weight, dry callus weight and per cent dry matter accumulation in the calli in all the eight genotypes during various passages of subculture (20-80 days) indicated that there was a marked variation in the response on these parameters as effected by different genotypes. In general, all the genotypes showed steady growth of callus upto 40-60 days and there was a initial increase in the dry matter accumulation upto 40 days followed by gradual decrease till 80 days of the callus growth. The optimum callus growth for all the three parameters steady was observed between 20 and 40 days of callus culture. Hence, the calli used for various experiments on differentiation and regeneration were taken from the stage of 20-40 days of callus growth in culture from all the three explants of the eight genotypes under study.

Fig.9 - Growth rate of calli as measured by per cent dry matter content over passages in callus from cotyledon explant of eight genotypes of lucerne



4.1.4. *In vitro* morphogenetic responses of callus differentiation and plant regeneration :

Based on the callus induction response and callus growth rate and callus quality, the calli from hypocotyl, epicotyl and cotyledon explants of all the eight genotypes of lucerne under study were selected for *in vitro* regeneration. The regeneration studies were conducted following two pathways:

(A) Morphogenetic response of calli directly on regeneration media, and

(B) Morphogenetic response of calli on the regeneration media following auxin shock treatment.

4.1.4.1 Morphogenetic response of calli directly on regeneration media:

The selected calli from various explants of all the genotypes were transferred to various regeneration media combinations based on MS and SH basal media with various combinations of plant growth regulators (auxins and cytokinins) to study their morphogenetic potential.

MS basal with growth regulators

MS basal medium with different concentration of auxins and cytokinins were used for studying morphogenetic response of the calli. In total forty-eight different MS basal based regeneration media supplemented with kinetin (0.0 mg/l – 8.0 mg/l), BAP (0.0 mg/l- 4.0 mg/l), NAA (0.0 mg/l- 1.0 mg/l) and IAA (0.0 mg/l- 1.0 mg/l) in various combinations were used. The calli selected on the basis of colour and texture were transferred to these media. The results are presented in table-4.12. When MS medium without any growth regulators was used, the calli from all the three explants hypocotyl, epicotyl and cotyledon of C-10, IG-1212, IL-75, A-3, LLC-9, Anand-2 AL-95-12 were turned brown within twenty days of their transfer without showing any morhogenetic differentiation. However, somatic embryos were induced from the callus of cotyledon explant of LLC-3 genotype without any auxin and cytokinin (Plate-7, Fig.1). These somatic embryos developed through various stages of

Table 4.12 - Morphogenetic response of calli on MS based regeneration media

S no	Basal media	Kin mg/l	BAP mg/l	NAA mg/l	IAA mg/l	Explant	Response	genotype
1	MS	0	0	0	0	H,Ep,C&C	Br, callus only &S.E.	1,2,3,4,5,7,8 & 6
2	MS	0.25	0	0	0	H,Ep,C	Lt yw, nod callus only	1,2,3,4,5,6,7,8,
3	MS	0.25	0	0.5	0	H,Ep,C	Lt y w, fri callus only	5,6,7
4	MS	0.25	0	1	0	H,Ep,C	Lt y w, fri callus only	5,6,7
5	MS	0.25	0	0	0.5	H,Ep,C	Lt y w, fri callus only	5,6,7
6	MS	0.25	0	0	1	H,Ep,C	Lt y w, lit br, fri callus only	1,2,3,4,5,6,7,8,
7	MS	0.5	0	0	0	H,Ep,C	Lt yw, nod callus only	1,2,3,4,5,6,7,8,
8	MS	0.5	0	0.5	0	H,Ep,C	Lt y w, g, differentiating callus only	1,2,3,4,5,6,7,8,
9	MS	0.5	0	1	0	H,Ep,C	Lt y w, g, differentiating callus only	1,2,3,4,5,6,7,8,
10	MS	0.5	0	0	0.5	H,Ep,C	Lt y w, fri callus only	5,6,7
11	MS	0.5	0	0	1	H,Ep,C	Lt y w, lit br, fri callus only	1,2,3,4,5,6,7,8,
12	MS	1	0	0	0	H,Ep,C	Lt yw, nod callus only	1,2,3,4,5,6,7,8,
13	MS	1	0	0.5	0	H,Ep,C	Lt yg, differentiating callus only	1,2,3,4,5,6,7,8,
14	MS	1	0	1	0	H,Ep,C	Lt yg, differentiating callus only	1,2,3,4,5,6,7,8,
15	MS	1	0	0	0.5	H,Ep,C	Lt yw,fri callus only	5,6,7
16	MS	1	0	0	1	H,Ep,C	Lt yw,fri callus only	5,6,7
17	MS	0	0.5	0	0	H,Ep,C	Lt wy, nod callus only	1,2,3,4,5,6,7,8,
18	MS	0	0.5	0.5	0	H,Ep,C	Lt yw, fri callus only	5,6,7
19	MS	0	0.5	1	0	H,Ep,C	Lt yw, fri callus only	5,6,7
20	MS	0	0.5	0	0.5	H,Ep,C	Lt yw, fri callus only	5,6,7
21	MS	0	0.5	0	1	H,Ep,C	Lt yw, lit br, fri callus only	1,2,3,4,5,6,7,8,
22	MS	0	1	0	0	H,Ep,C	Lt yw, nod callus only	1,2,3,4,5,6,7,8,
23	MS	0	1	0.5	0	H,Ep,C	Lt yw, fri callus only	5,6,7
24	MS	0	1	1	0	H,Ep,C	Lt yw, fri callus only	5,6,7
25	MS	0	1	0	0.5	H,Ep,C	Lt yw, fri callus only	5,6,7
26	MS	0	1	0	1	H,Ep,C	Lt yw, lit br, fri callus only	1,2,3,4,5,6,7,8,
27	MS	0.5	0.5	0	0	H,Ep,C	Lt yg, differentiating callus only	1,2,3,4,5,6,7,8,
28	MS	0.5	0.5	0.5	0	H,Ep,C	Lt yg, differentiating callus only	1,2,3,4,5,6,7,8,
29	MS	0.5	0.5	1	0	H,Ep,C	Lt yg, differentiating callus only	1,2,3,4,5,6,7,8,
30	MS	0.5	0.5	0	0.5	H,Ep,C	Lt yw, gw,differentiating callus only	1,2,3,4,5,6,7,8,
31	MS	0.5	0.5	0	1	H,Ep,C	Lt yw, gw,differentiating callus only	1,2,3,4,5,6,7,8,
32	MS	0.5	1	0	0	H,Ep,C	Lt yw, gw,differentiating callus only	1,2,3,4,5,6,7,8,
33	MS	0.5	1	0.5	0	H,Ep,C	Lt yw, gw,differentiating callus only	1,2,3,4,5,6,7,8,
34	MS	0.5	1	1	0	H,Ep,C	Lt yw, gw,differentiating callus only	1,2,3,4,5,6,7,8,
35	MS	0.5	1	0	0.5	H,Ep,C	Lt yw, gw,differentiating callus only	1,2,3,4,5,6,7,8,
36	MS	0.5	1	0	1	H,Ep,C	Lt yw, gw,differentiating callus only	1,2,3,4,5,6,7,8,
37	MS	2	0	1	0	H,Ep,C	G,lit br, & yg,fri callus only	2,3
38	MS	2	0	0	1	H,Ep,C	G,lit br, & yg,fri callus only	2,3
39	MS	2	0	0.5	0.5	H,Ep,C	G,lit br, & yg,fri callus only	2,3
40	MS	0	2	1	0	H,Ep,C	Lt yw, nod callus only	1,2,3,4,5,6,7,8,
41	MS	0	2	0.5	0.5	H,Ep,C	Lt yw, nod callus only	1,2,3,4,5,6,7,8,
42	MS	4	0	1	0	H,Ep,C	Yg, differentiating callus only	1,2,3,4,5,6,7,8,
43	MS	4	1	0.5	0.5	H,Ep,C	Yg, differentiating callus only	1,2,3,4,5,6,7,8,
44	MS	4	2	0.5	0.5	H,Ep,C	Yg, differentiating callus only	1,2,3,4,5,6,7,8,
45	MS	6	0	0.5	0.5	H,Ep,C	Yg, differentiating callus only	1,2,3,4,5,6,7,8,
46	MS	8	0	0.5	0.5	H,Ep,C	Yg, differentiating callus only	1,2,3,4,5,6,7,8,
47	MS	0	3	0.5	0.5	H,Ep,C	Lt yw, nod callus only	1,2,3,4,5,6,7,8,
48	MS	0	4	0.5	0.5	H,Ep,C	Lt yw, nod callus only	1,2,3,4,5,6,7,8,

Abbreviations

Explants	Genotypes	Colour
H- hypocotyl	1 C-10	w- white
Ep- epicotyl	2 IG1212	g- green
C- cotyledon	3 IL-75	Lit br- little bit of brown
	4 A-3	y- yellowish
	5 LLC-9	nod- nodular
	6 LLC-3	fri- friable
	7 Anand-2	
	8 AL-95-12	

somatic embryogenesis. Light yellowish white and nodular types calli were observed in MS medium with 0.25 mg/l kinetin or 0.5 mg/l kinetin or 1.0 mg/l kinetin or 0.5BAP mg/l or 1.0 mg/l BAP or 2.0 mg/l BAP +1.0 NAA mg/l or 2.0 BAP mg/l +0.5 mg/l NAA+0.5 mg/l IAA or 3.0 mg/l BAP+0.5 mg/l IAA +0.5 mg/l NAA or 4.0 mg/l BAP+0.5 mg/l NAA +0.5 mg/l IAA from the calli of hypocotyl, epicotyl and cotyledon explants in C-10, IG-1212, IL-75, A-3, LLC-9, LLC-3 Anand-2 and AL-95-12. Light yellowish white and friable types of calli were formed in MS medium with 0.25 mg/l kinetin +0.5 mg/l NAA or 0.25 mg/l kinetin+1.0 mg/l NAA or 0.25 mg/l kin+0.5 mg/l IAA or 0.5 mg/l kin +0.5 mg/l IAA or 1.0 mg/l kin +0.5 mg/l IAA or 1.0 mg/l kin + 1.0 mg/l IAA or 0.5 mg/l BAP +0.5 mg/l NAA or 0.5 mg/l BAP + 1.0 mg/l NAA or 0.5 mg/l BAP + 0.5 mg/l IAA or 1.0 mg/l BAP +0.5 mg/l NAA or 1.0 mg/l BAP + 1.0 mg/l NAA or 1.0 mg/l BAP + 0.5 mg/l IAA from the calli of hypocotyl, epicotyl and cotyledon explants in LLC-9, LLC-3 and Anand-2. Light yellowish white and little bit of brown, friable callus was found in MS medium with 0.25 mg/l kin + 1.0 mg/l IAA or 0.5 mg/l kin + 1.0 mg/l IAA or 0.5 mg/l BAP + 1.0 mg/l IAA or 1.0 mg/l BAP + 1.0 mg/l IAA from hypocotyls, epicotyl and cotyledon explants of C-10, IG-1212, IL-75, A-3, LLC9, LLC-3, Aanad-2 and AL-95-12. Light yellowish white, green and differentiating types of calli were observed in MS medium with 0.5 mg/l kin+ 0.5 mg/l NAA or 0.5 mg/l kin + 1.0 mg/l NAA from the calli of hypocotyl, epicotyl and cotyledon explants in C-10, IG-1212, IL-75, A-3, LLC-9, LLC-3, Anand-2 and AL-95-12. Light yellowish green and differentiating types of calli were formed in MS medium with 1.0 mg/l kin +0.5 mg/l NAA or 1.0 mg/l kin +1.0 mg/l NAA or 0.5 mg/l kin + 0.5 mg/l BAP or 0.5 mg/l kin + 0.5 mg/l BAP + 0.5 mg/l NAA or 0.5 mg/l kin + 0.5 mg/l BAP + 1.0 mg/l NAA from the calli of hypocotyl, epicotyl and cotyledon explants in C-10, IG-1212, IL-75, A-3 LLC-9, LLC-3, Anand-2 and AL –95-12. Light yellowish white, greenish white and differentiating types of calli were formed in MS medium with 0.5 mg/l kin +0.5 mg/l BAP +0.5 mg/l IAA or 0.5 mg/l kin +0.5 mg/l BAP +1.0 mg/l IAA or 0.5 mg/l kin +1.0 mg/l BAP or 0.5 mg/l kin +1.0 mg/l BAP + 0.5 mg/l NAA or 0.5 mg/l kin + 1.0 mg/l BAP +1.0 mg/l NAA or 0.5 mg/l kin + 1.0 mg/l BAP + 0.5 mg/l IAA or 0.5 mg/l kinetin+ 1.0 mg/l BAP + 1.0 mg/l IAA from the calli of hypocotyl, epicotyl and cotyledon explants of C-10, IG-1212,

IL-75, A-3, LLC-9, LLC-3, Anand-2, AL-95-12. Green little bit of brown and yellowish green and friable types of calli were observed in MS medium with 2.0 mg/l kinetin+ 1.0 mg/l NAA or 2.0 mg/l kinetin + 1.0 mg/l IAA or 2.0 mg/l kinetin +0.5 mg/l NAA +0.5 mg/l IAA from the calli of hypocotyl, epicotyl and cotyledon explants of IG-1212 and IL-75. Yellowish green and differentiating types of callus was observed in MS medium with 4.0 mg/l kin +1.0 mg/l NAA or 4.0 mg/l kin + 1.0 mg/l BAP +0.5 mg/l NAA + 0.5 mg/l IAA or 4.0 mg/l kin + 2.0 mg/l BAP +0.5 mg/l NAA + 0.5 mg/l IAA or 6.0 mg/l kin +0.5 mg/l IAA + 0.5 mg/l NAA or 8.0 mg/l kin +0.5 mg/l IAA +0.5 mg/l NAA from the calli of hypocotyl, epicotyl and cotyledon explants of C-10, IG-1212, IL-75, A-3, LLC-9, LLC-3, Anand-2, AL-95-12.In all these media combinations based on MS basal medium, it was only the MS basal medium without any growth regulators supplementation on which the development of somatic embryo occurred from the callus of cotyledon explant of LLC-3 genotype. There was no differentiation observed for shoot bud organogenesis or embryogenesis from the calli of various explants of different genotypes on any other media combinations.

SH basal with growth regulators

SH basal medium with different combinations of auxins and cytokinins were used for studying morphogenetic response of the calli In total 48 different SH basal based regeneration media supplemented with kinetin (0.0 mg/l- 8.0 mg/l), BAP (0.0 mg/l-4.0 mg/l), NAA (0.0mg/l-1.0 mg/l) and IAA (0.0 mg/l-1.0 mg/l) in various combinations as in case with MS basal, were also used. The calli selected on the basis of colour and texture were transferred to these media and incubated for 20-21 days in cultures. The results on different regeneration media combinations using SH as basal medium are presented in Table-4.13. The SH medium without any growth regulators when inoculated with the calli selected for regeneration from the hypocotyl, epicotyl and cotyledon explants of all the eight genotypes turned brown within twenty days and showed no differentiation. Light yellowish white and nodular types of calli were observed in SH medium with 0.25 mg/l kin or 0.25 mg/l kin + 0.5 mg/l NAA or 0.25 mg/l kin + 1.0 mg/l NAA or 0.5 mg/l kinetin or 0.5 mg/l kin +0.5 mg/l IAA or 0.5 mg/l BAP or 1.0 mg/l BAP or 1.0 mg/l BAP + 0.5 mg/l

Table 4.13 - Morphogenetic response of calli on SH based regeneration media

S. no.	Basal media	Kin mg/l	BAP mg/l	NAA mg/l	IAA mg/l	Explant	Response	Genotypes
1	SH	0	0	0	0	H, Ep, C	Br, callus only	1,2,3,4,5,6,7,8
2	SH	0.25	0	0	0	H, Ep, C	Lt y w, nod callus only	2,3
3	SH	0.25	0	0.5	0	H, Ep, C	Lt y w, nod callus only	2,3
4	SH	0.25	0	1	0	H, Ep, C	Lt y w, nod callus only	2,3
5	SH	0.25	0	0	0.5	H, Ep, C	Lt y w, fri callus only	3,5
6	SH	0.25	0	0	1	H, Ep, C	Lt y w, fri callus only	3,5
7	SH	0.5	0	0	0	H, Ep, C	Lt yw, nod callus only	2,3
8	SH	0.5	0	0.5	0	H, Ep, C	Lt y g, differentiating callus only	6,7,8
9	SH	0.5	0	1	0	H, Ep, C	Lt y g, differentiating callus only	6,7,8
10	SH	0.5	0	0	0.5	H, Ep, C	Lt y w, nod callus only	2,3
11	SH	0.5	0	0	1	H, Ep, C	Lt y w, fri callus only	3, 5
12	SH	1	0	0	0	H, Ep, C	Lt yg, fri callus only	2,3,4,
13	SH	1	0	0.5	0	H, Ep, C	Lt yg,fri callus only	2,3,4,
14	SH	1	0	1	0	H, Ep, C	Lt yg, fri, callus only	2,3,4,
15	SH	1	0	0	0.5	H, Ep, C	Lt yw,differentiating callus only	4, 5
16	SH	1	0	0	1	H, Ep, C	Lt yw,differentiating callus only	4, 5
17	SH	0	0.5	0	0	H, Ep, C	Lt wy, nod callus only	2, 3
18	SH	0	0.5	0.5	0	H, Ep, C	Lt y, fri callus only	4,5,6,7,8,
19	SH	0	0.5	1	0	H, Ep, C	Lt y, fri callus only	4,5,6,7,8,
20	SH	0	0.5	0	0.5	H, Ep, C	Lt y, fri callus only	4,5,6,7,8,
21	SH	0	0.5	0	1	H, Ep, C	Lt y, fri callus only	4,5,6,7,8,
22	SH	0	1	0	0	H, Ep, C	Lt yw, nod callus only	2,3
23	SH	0	1	0.5	0	H, Ep, C	Lt yw, nod callus only	2,3
24	SH	0	1	1	0	H, Ep, C	Lt yw, nod callus only	2,3
25	SH	0	1	0	0.5	H, Ep, C	Lt yw, fri callus only	3, 5
26	SH	0	1	0	1	H, Ep, C	Lt yw, lit br, fri callus only	1
27	SH	0.5	0.5	0	0	H, Ep, C	Lt yg, differentiating callus only	1,2,3,4,5,6,7,8
28	SH	0.5	0.5	0.5	0	H, Ep, C	Lt yg, differentiating callus only	1,2,3,4,5,6,7,8
29	SH	0.5	0.5	1	0	H, Ep, C	Lt yg, differentiating callus only	1,2,3,4,5,6,7,8
30	SH	0.5	0.5	0	0.5	H, Ep, C	Lt yw, gw,differentiating callus only	1,2,3,4,5,6,7,8
31	SH	0.5	0.5	0	1	H, Ep, C	Lt yw, gw,differentiating callus only	1,2,3,4,5,6,7,8
32	SH	0.5	1	0	0	H, Ep, C	Lt yw, gw,differentiating callus only	1,2,3,4,5,6,7,8
33	SH	0.5	1	0.5	0	H, Ep, C	Lt yw, gw,differentiating callus only	1,2,3,4,5,6,7,8
34	SH	0.5	1	1	0	H, Ep, C	Lt yw, gw,differentiating callus only	1,2,3,4,5,6,7,8
35	SH	0.5	1	0	0.5	H, Ep, C	Lt yw, gw,differentiating callus only	1,2,3,4,5,6,7,8
36	SH	0.5	1	0	1	H, Ep, C	Lt yw, gw,differentiating callus only	1,2,3,4,5,6,7,8
37	SH	2	0	1	0	H, Ep, C	Lt y w, nod callus only	2, 3
38	SH	2	0	0	1	H, Ep, C	Lt y w, nod callus only	2, 3
39	SH	2	0	0.5	0.5	H, Ep, C	Lt y w, nod callus only	2, 3
40	SH	0	2	1	0	H, Ep, C	Lt y w, nod callus only	2, 3
41	SH	0	2	0.5	0.5	H, Ep, C	Lt y w, nod callus only	2, 3
42	SH	4	0	1	0	H, Ep, C	Lt y w, gw, differentiating callus only	1,2,3,4,5,6,7,8
43	SH	4	1	0.5	0.5	H, Ep, C	Lt y w, gw, differentiating callus only	1,2,3,4,5,6,7,8
44	SH	4	2	0.5	0.5	H, Ep, C	Lt y w, gw, differentiating callus only	1,2,3,4,5,6,7,8
45	SH	6	0	0.5	0.5	H, Ep, C	Lt y w, gw, differentiating callus only	1,2,3,4,5,6,7,8
46	SH	8	0	0.5	0.5	H, Ep, C	Lt y w, gw, differentiating callus only	1,2,3,4,5,6,7,8
47	SH	0	3	0.5	0.5	H, Ep, C	Lt yw, fri callus only	3,5
48	SH	0	4	0.5	0.5	H, Ep, C	Lt yw, fri callus only	3,5

Abbreviations

Explants	Genotypes	Colour
H- hypocoty	1 C-10	w- white
Ep- epicotyl	2 IG1212	g- green
C- cotyledor	3 IL-75	Lit br- little bit of brown
	4 A-3	y- yellowish
	5 LLC-9	nod- nodular
	6 LLC-3	fri- friable
	7 Anand-2	
	8 AL-95-12	

NAA or 1.0 mg/l BAP + 1.0 mg/l NAA or 2.0 mg/l kin + 1.0 mg/l NAA or 2.0 mg/l kin +1.0 mg/l IAA or 2.0 mg/l kin + 0.5 mg/l NAA +0.5 mg/l IAA or 2.0 mg/l BAP+ 1.0 mg/l NAA or 2.0 mg/l BAP +0.5 mg/l NAA +0.5 mg/l IAA from the calli of hypocotyl, epicotyl and cotyledon explants of IG-1212 and IL-75. Light yellowish white and friable calli were formed in SH medium with 0.25 mg/l Kin + 0.5 mg/l IAA or 0.25 mg/l kin + 1.0 mg/l IAA or 0.5 mg/l kin +1.0 mg/l IAA or 1.0 mg/l BAP +0.5 mg/l IAA or 3.0 mg/l BAP + 0.5 mg/l IAA + 0.5 mg/l NAA or 4.0 mg/l BAP + 0.5 mg/l IAA + 0.5 mg/l BAP from the calli of hypocotyl, epicotyl and cotyledon explants of IL-75 and LLC-9. Light yellowish green and differentiating calli were found in SH medium with 0.5 mg/l kin + 0.5 mg/l NAA or 0.5 mg/l kin + 1.0 NAA mg/l or 0.5 mg/l kin + 0.5 mg/l BAP or 0.5 mg/l kin +0.5 mg/l BAP + 0.5 mg/l NAA or 0.5 mg/l kin + 0.5 mg/l BAP + 1.0 mg/l NAA from the calli of hypocotyl, epicotyl and cotyledon explants of LLC-3, Anand-2 and AL-95-12. Light yellowish green and friable calli were observed in SH medium with 1.0 mg/l kin or 1.0 mg/l kin + 0.5 mg/l NAA or 1.0 mg/l kin + 1.0 mg/l NAA from the calli of hypocotyl, epicotyl and cotyledon explants from IG-1212, IL-75, A-3. Light yellowish white and differentiating calli were found in SH medium with 1.0 mg/l kin + 0.5 mg/l IAA or 1.0 mg/l kin + 1.0 mg/l IAA from the calli of hypocotyl, epicotyl and cotyledon explants from A-3, LLC-9. Light yellow and friable callus was formed in SH medium with 0.5 mg/l BAP + 0.5 mg/l NAA or 0.5 mg/l BAP + 1.0 mg/l NAA or 0.5 mg/l BAP + 0.5 mg/l IAA or 0.5 mg/l BAP + 1.0 mg/l IAA from the calli of hypocotyl, epicotyl and cotyledon explants from A-3, LLC-9, LLC-3, Anand-2, AL-95-12. Light yellowish white, greenish white and differentiating types of calli were observed in SH medium with 0.5 mg/l kin + 0.5 mg/l BAP+ 0.5 mg/l IAA or 0.5 mg/l kin + 0.5 mg/l BAP + 1.0 mg/l IAA or 0.5 mg/l kin + 1.0 mg/l BAP or 0.5 mg/l kin + 1.0 mg/l BAP + 0.5 mg/l NAA or 0.5 mg/l kin + 1.0 mg/l BAP + 1.0 mg/l NAA or 0.5 mg/l kin + 1.0 mg/l BAP + 0.5 mg/l IAA or 0.5 mg/l kin + 1.0 mg/l BAP + 1.0 mg/l IAA or 4.0 mg/l kin + 1.0 mg/l NAA or 4.0 mg/l kin + 1.0 mg/l BAP + 0.5 mg/l NAA + 0.5 mg/l IAA or 4.0 mg/l kin + 2.0 mg/l BAP + 0.5 mg/l NAA + 0.5 mg/l IAA or 6.0 mg/l kin + 0.5 mg/l NAA + 0.5 mg/l IAA or 8.0 mg/l kin + 0.5 mg/l NAA + 0.5 mg/l IAA from the calli of hypocotyl, epicotyl and cotyledon explants of C-10, IG-1212, IL-75, A-3, LLC-9 LLC-3, Anand-2, AL-95-

12. Light yellowish white and little bit of brown and friable types of calli were formed in SH medium with 1.0 mg/l BAP +1.0 mg/l IAA from the calli of hypocotyl, epicotyl and cotyledon explants of C-10. However, there was no differentiation observed for somatic embryogenesis or shoot bud organogenesis in these media combinations from the calli of various explants of different genotypes.

4.1.4.2. Morphogenetic response of calli on regeneration media following auxin shock treatment :

The selected calli from different explants of various genotypes under study were transferred on auxin shock treatment medium for four days following the method of Romagnoli *et al.* (1996) and then transferred subsequently on four different regeneration media namely SHKI, MSKI, SHPKI and MSPKI sequences, separately for regeneration.

The calli grown on SH media regeneration sequence (SHKI) or MS regeneration sequence (MSKI) after the auxin shock treatment did not show any kind of regeneration response. In these two regeneration media sequences the calli from different explants of various genotypes developed into various types of quality of calli ranging from light yellowish white, yellowish green, light green and yellowish brown. The effect of basal media was evident on the calli as greening of the calli was observed in MSKI regeneration media sequence whereas the calli turned yellowish brown and became non regenerative on the SHKI regeneration media sequence

After auxin treatment when the calli were transferred to proline supplemented MS or SH media, globular shaped somatic embryos were visible within 6-7 days of cultures. The cultures were full of these globular structures (Plate-7, Fig.2) in 18-20 days of cultures. These globular shaped structures remained arrested at this stage only and there was no change in the callus colour. When these calli were transferred to kinetin and IAA supplemented MS or SH (MSPKI and SHPKI) the effect of basal medium became evident and greening of callus was observed in MSPKI

Plate - 7

Somatic embryogenesis

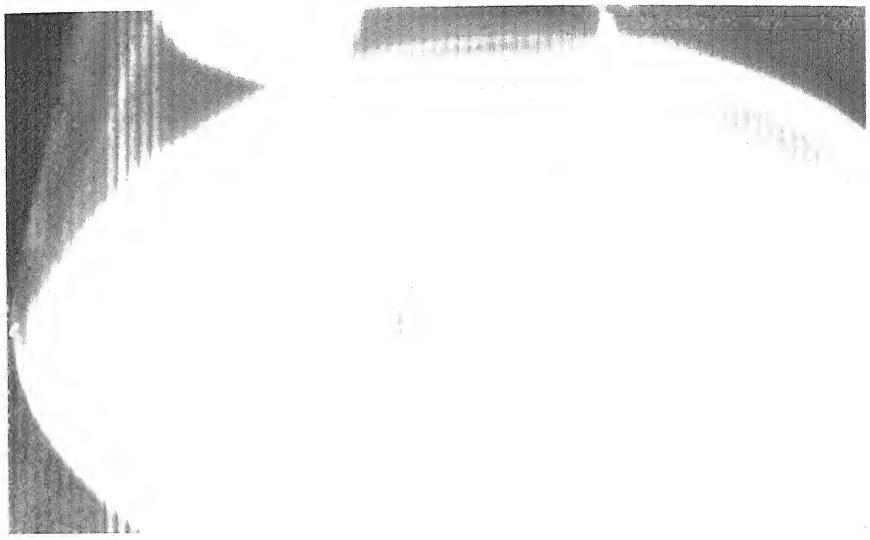


Fig.1 - Somatic embryos
(Direct on regeneration medium)
Se - Somatic embryo

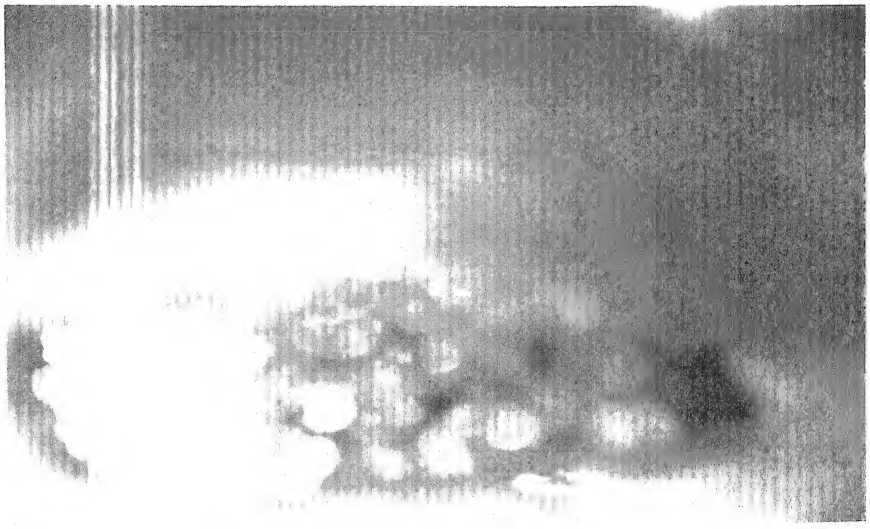


Fig.2 - Globular somatic embryos
(On MSPK1 after auxin shock)

regeneration sequence while calli on SHPKI regeneration sequence remained yellowish and became brownish. The calli of all the genotypes developed shoot primordial on 4.0 mg/l kinetin and 1.0 mg/l IAA step of MSPKI regeneration sequence. On further increasing the kinetin concentration to 8.0 mg/l in the next step of regeneration sequence, shoot formation was observed in calli of hypocotyl explant of IL-75, C-10, IG-1212, AL-95-12, cotyledon explant of A-3 and LLC-3 and epicotyl explant of IL-75. On this high concentration of kinetin the callus accompanying the shoots became yellowish brown and was transferred to reduced kinetin concentration (2.0 mg/l). Shoot bud multiplication was observed in this step. Development of shoots was observed on plain MS medium.

In MSPKI regeneration sequence the shoot formation occurred mostly through shoot bud organogenesis on kinetin supplemented media. The proline supplementation and effect of basal media were found critical as regeneration occurred on proline supplemented MSPKI regeneration media sequence only and no regeneration was observed on MSKI or SHKI sequences which were devoid of proline.

The response for regeneration varied among different explants and the genotypes. Regeneration though shoot bud organogenesis following MSPKI sequence of media was observed form the calli of different explants in six genotypes only (Table -4.14) at varying frequencies. The efficiency of regeneration as reflected by number of shoots per calli was maximum from the calli of cotyledon explant of LLC-3 genotype (23.25 shoots/callus) followed by hypocotyl explant of IL-75 genotype (15.0 shoots/callus). The minimum efficiency for regeneration (9.0 shoots/calli) was observed from the calli of epicotyl and cotyledon explants of IL-75 and A-3 genotypes, respectively. Shoot bud formation from calli of cotyledon explant occurred in LLC-3 and A-3 genotypes only (Plate-8a, Fig.1&2). The shoot bud regeneration from calli of hypocotyl explant was exhibited in IL-75, IG-1212, AL-95-12 and C-10 genotypes only (Plate-8a, Fig.3; Plate-8b, Fig.4-7) and from epicotyl it occurred in IL-75 only (Plate-8b, Fig.7). While regeneration was exhibited from the calli of two out of three explants in IL-75, it occurred form the calli of only one out of three explants in LLC-3, IG-1212, AL-95-12, C-10 and A-3

Table 4.14 : Frequency of shoot bud organogenesis

Genotypes	Explants	Number of shoots/calli
LLC-3	Hypocotyl	0.0
	Epicotyl	0.0
	Cotyledon	23.25
LLC-9	Hypocotyl	0.00
	Epicotyl	0.0
	Cotyledon	0.0
IL-75	Hypocotyl	15.00
	Epicotyl	9.00
	Cotyledon	0.0
A-3	Hypocotyl	0.0
	Epicotyl	0.0
	Cotyledon	9.0
C-10	Hypocotyl	10.0
	Epicotyl	0.0
	Cotyledon	0.0
IG-1212	Hypocotyl	12.0
	Epicotyl	0.0
	Cotyledon	0.0
Anand-2	Hypocotyl	0.0
	Epicotyl	0.0
	Cotyledon	0.0
AL-95-12	Hypocotyl	12.0
	Epicotyl	0.0
	Cotyledon	0.0

genotypes. There was no regeneration from any explant from Anand-2 and LLC-9 genotypes. The calli form hypocotyl showed maximum response for regeneration followed by cotyledon and the least response for regeneration was exhibited from the calli of epicotyl explant from various genotypes. The shoot buds regenerated from the calli of various explants in the above mentioned six genotypes further developed and grown as shoots (Plate-13a & 13b, Fig.1-7)

4.1.4.3. Histology of morphogenetic responses

Morphogenetic potential towards regenerability of different types of calli was observed and it was found that only whitish or greenish white and granular or nodular types of calli were capable of regeneration. It was observed that the cell mass among the calli potentially capable of differentiation were composed of compact round shaped cells of comparatively small and uniform size with densely staining cytoplasm (Plate-9, Fig.1). On the other hand, the vitrious and other types of non regenerating calli were mostly composed of loose mass of cells having various shapes and sizes ranging from small ovoid to large vesicular, tubular or syphonous cells with feebly stainable cytoplasm and obscure nucleus (Plate-9, Fig.2).

The regenerating calli when induced for differentiation on various combinations of growth regulators in regenerating media exhibited both pathways of regeneration, *i.e., via* somatic embryogenesis and the shoot bud organogenesis through somatic embryogenesis was quite ephemeral. Histological examination for occurrence of somatic embryogenesis and shoot bud organogenesis was carried out under the compound microscope after fixing, microtome sectioning and staining with safranin-fastgreen of the regenerating calli in which differentiation was initiated. In the microtomed sections of such calli, the developing somatic embryos exhibited bipolar meristematic structures with a plumule region at the distal end and redical region at the proximal end of the structure which was embeded in the callus mass (Plate-10, Fig.1). Such somatic embryos were largely globular (Plate-10, Fig.2) heart shaped

(Plate-11, Fig.1) or torpedo shaped having cotyledonary protuberances (Plate-11, Fig.2) when examined under the stereoscopic microscope from a fresh callus mass.

The most commonly occurring and stable morphogenetic response towards differentiation from the regenerating calli was through somatic bud organogenesis. Histological examination of such regenerating calli revealed that unipolar meristematic outgrowths started appearing on the surface of these calli during organogenesis (Plate-12, Fig.1). These outgrowths soon assumed the structure of vegetative shoot buds and the typical shoot bud structure having clearly discernible tunica-corpus organization with subtending leaf primordia developed on the surface of differentiating calli (Plate-12, Fig.2&3). Such shoot buds developed into vegetative shoots bearing leaves laterally which were like stumps which had did not bear roots.

4.1.4.4. Rhizogenesis

The shoots regenerated on MSPKI regeneration medium sequence from the calli of various explants in six genotypes, LLC-3, IL-75, A-3, C-10, IG-1212 and AL-95-12 lacked roots, possessing only stumps at their basal ends which were surrounded by the callus from which they arose. These shoots at early stage (1.0-2.0 cm long) along with little bit of surrounding callus were transferred on the fresh medium of the same composition (MS basal without any growth hormone). Root induction was observed at the base of the developing shoots in LLC-3 and IL-75 genotypes only after 15-20 days of culture, but the root development was poor even upto 30 days of culture and drooping of the leaves from most of the shoots started occurring in the plantlets in 30-35 days old cultures. These plantlets had poorly developed roots and did not survive when subjected to hardening process. This suggested the need to transfer the developing shoots on the root induction media for proper root development.

Eight different rooting media, four each based on MS and SH basal salts supplemented with 0.05 mg/l kinetin, 2.0 mg/l NAA, 2.0 and 5.0 mg/l IAA and with

Plate - 8a

Regeneration in different Genotypes

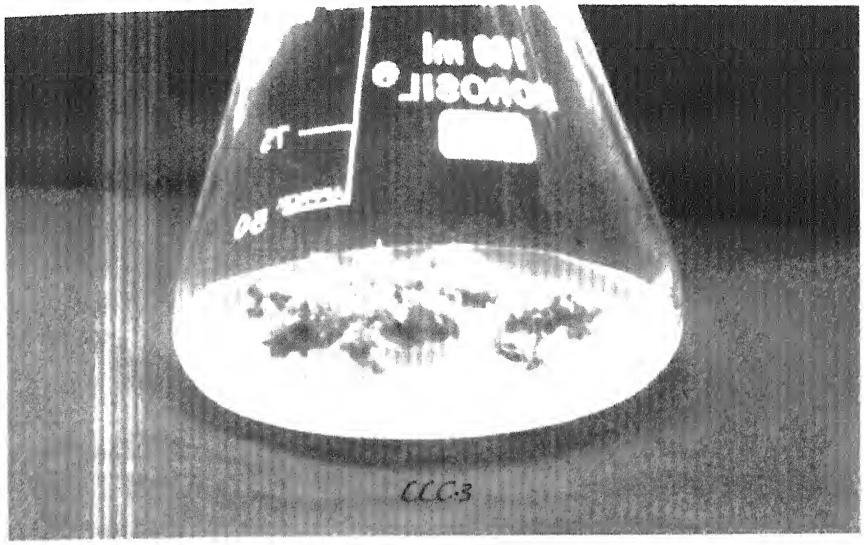


Fig.1 - Cotyledon

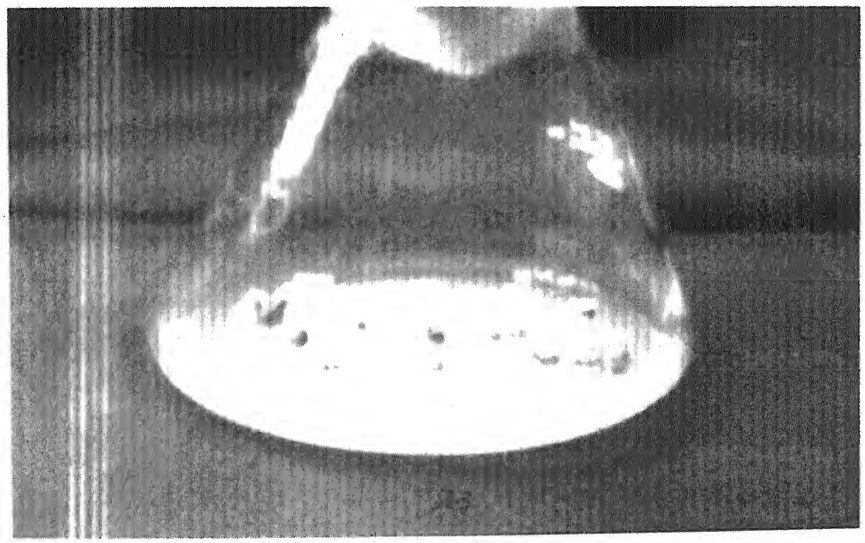


Fig.2 - Cotyledon

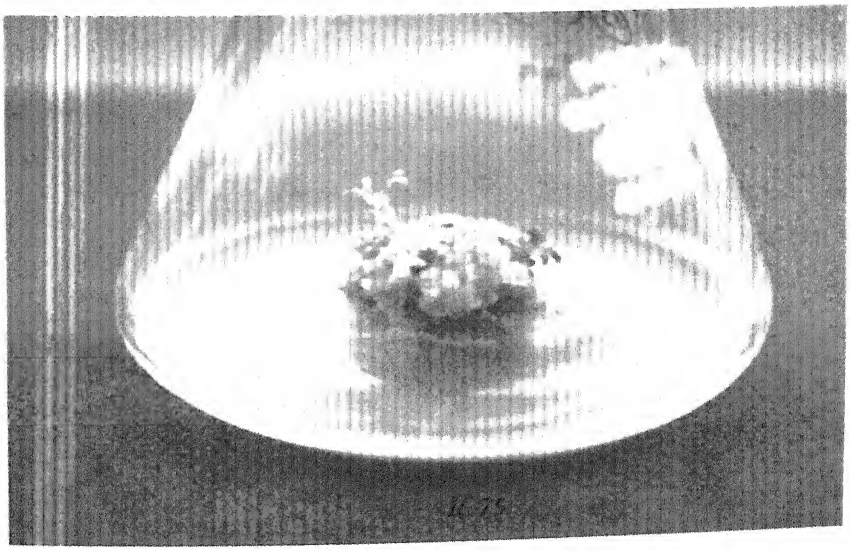


Fig.3 - Hypocotyl

Plate - 8b

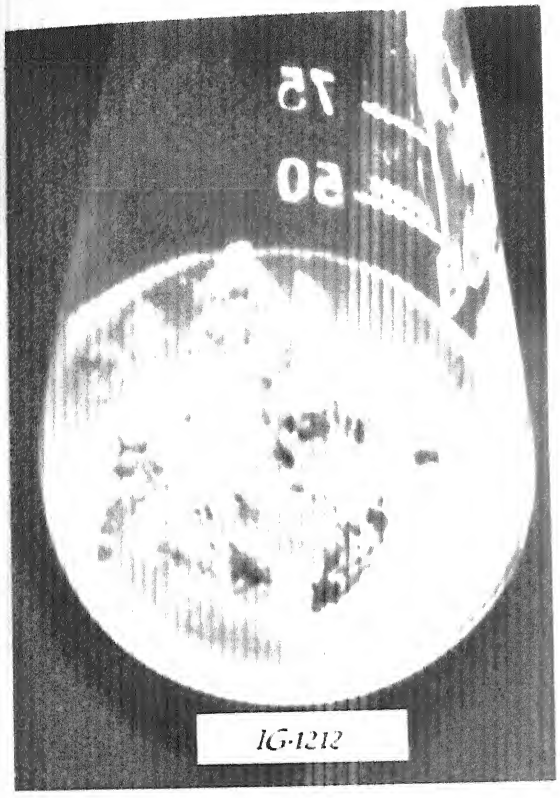


Fig.4 - Hypocotyl

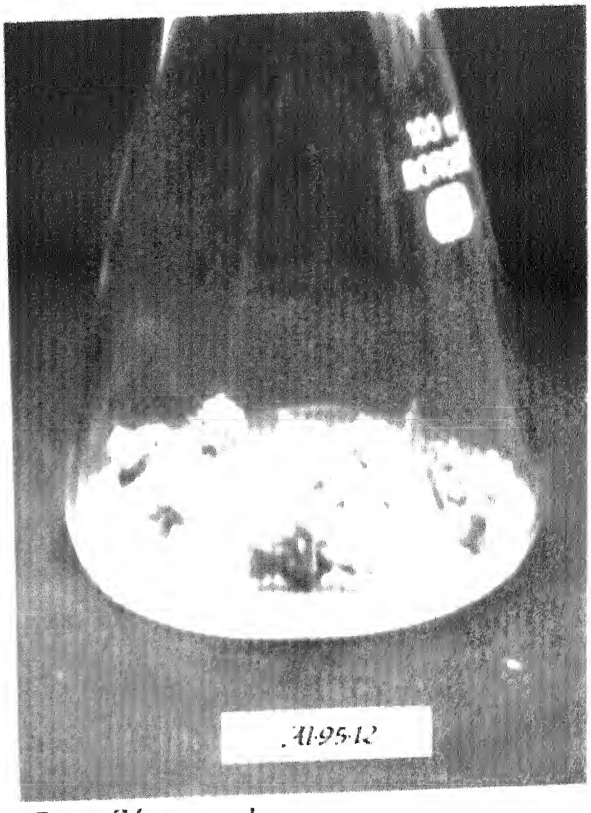


Fig.5 - Hypocotyl

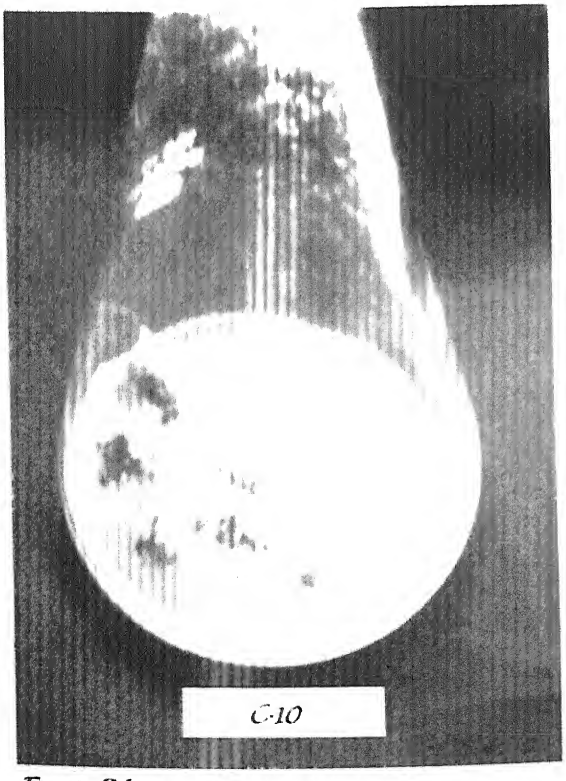


Fig.6 - Hypocotyl

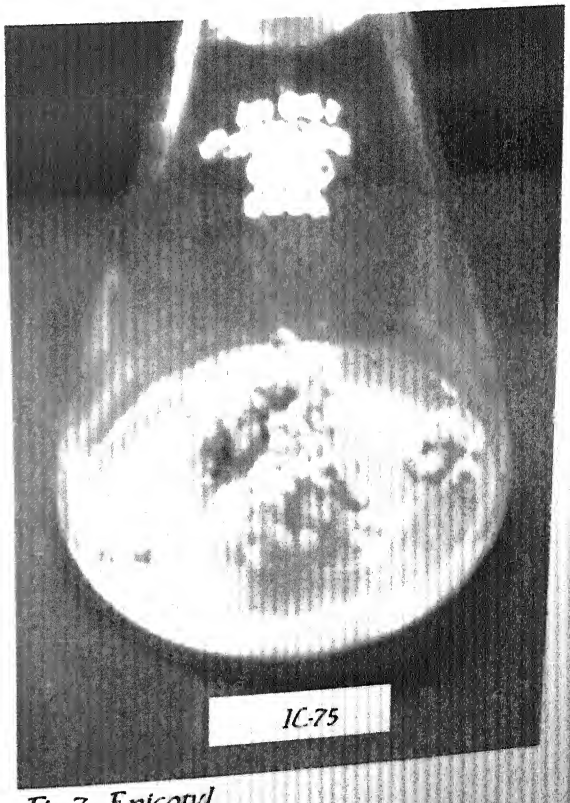


Fig.7 - Epicotyl

Plate - 9

Histology

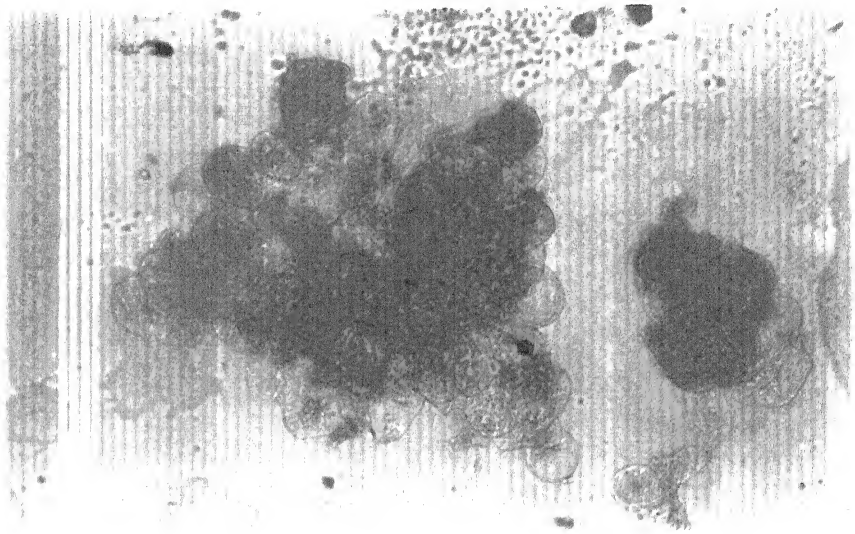


Fig.1 - Cell mass-Regenerating callus

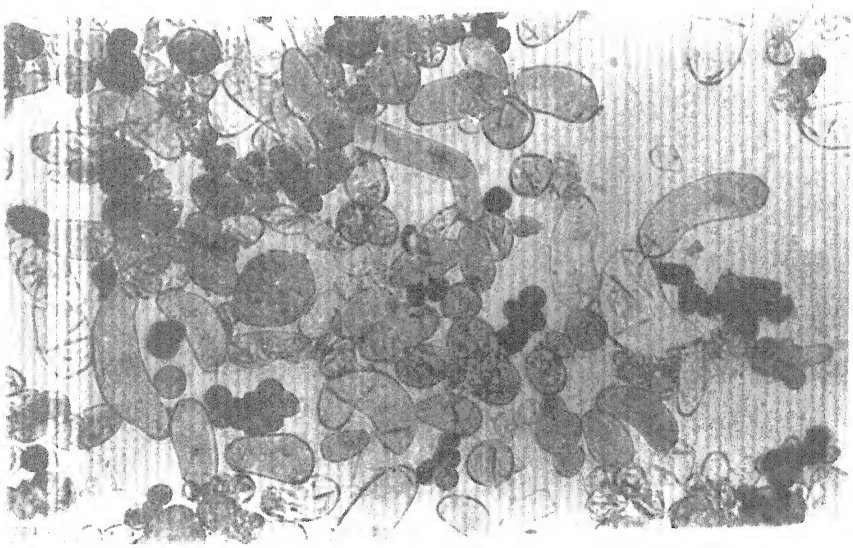


Fig.2 - Cell mass- Nonregenerating callus

Plate - 10

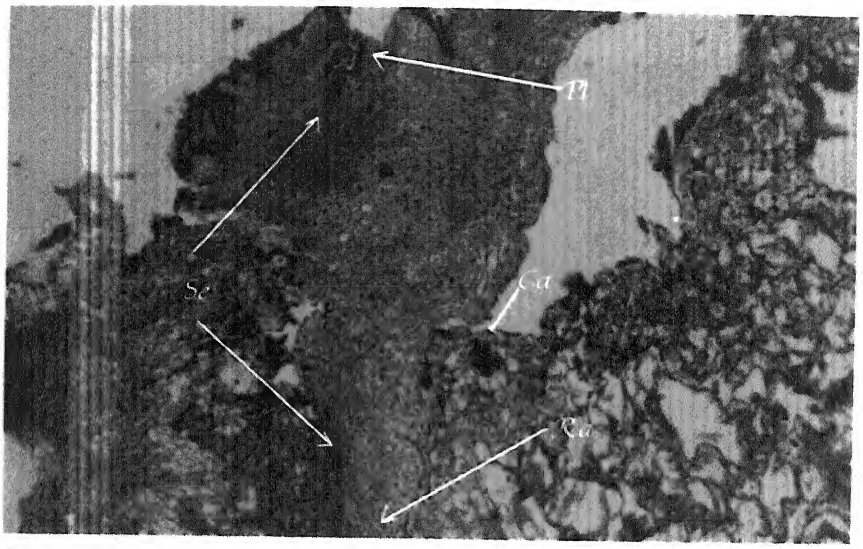


Fig.1 - Somatic embryo (Histology)

Ca - Callus
Pl - Plumule
Ra - Radicle
Se - Somatic embryo

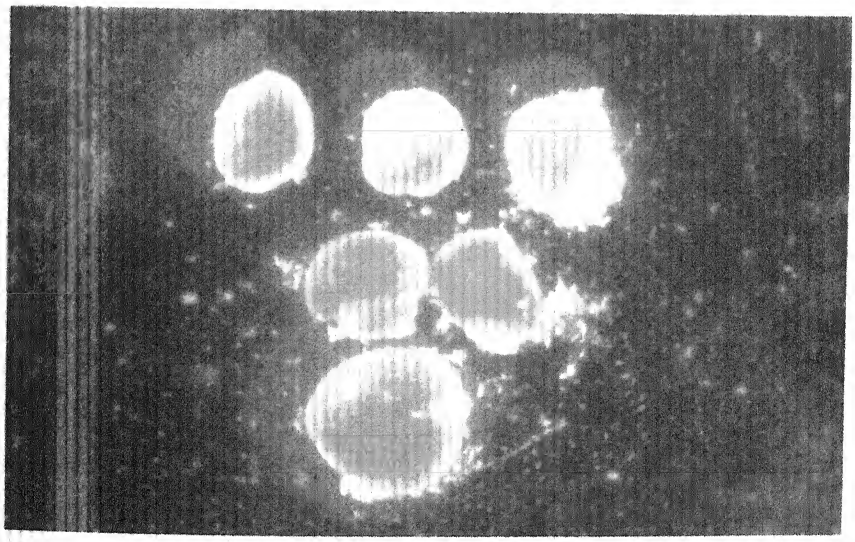


Fig.2 - Globular Somatic Embryos

Plate - 11

Somatic embryo development

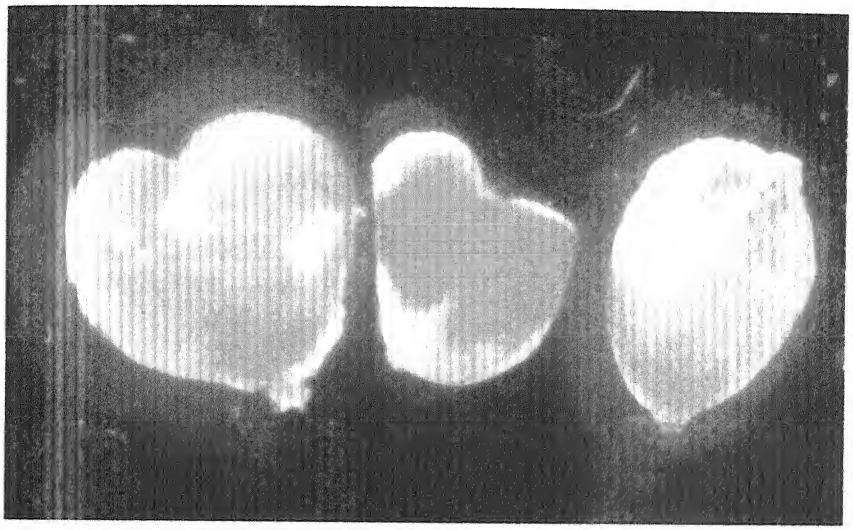


Fig.1 - Heart shaped Somatic embryos

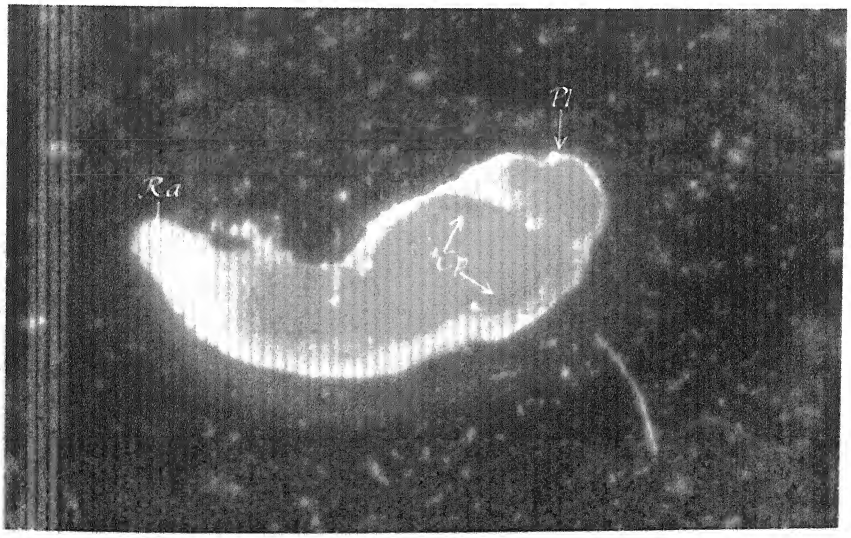


Fig.2 - Torpedo shaped Somatic Embryo

Cp - cotyledonary primordia
Pl - Plumule
Ra - Radicle

Plate - 12

Histology (Shoot Bud Organogenesis)

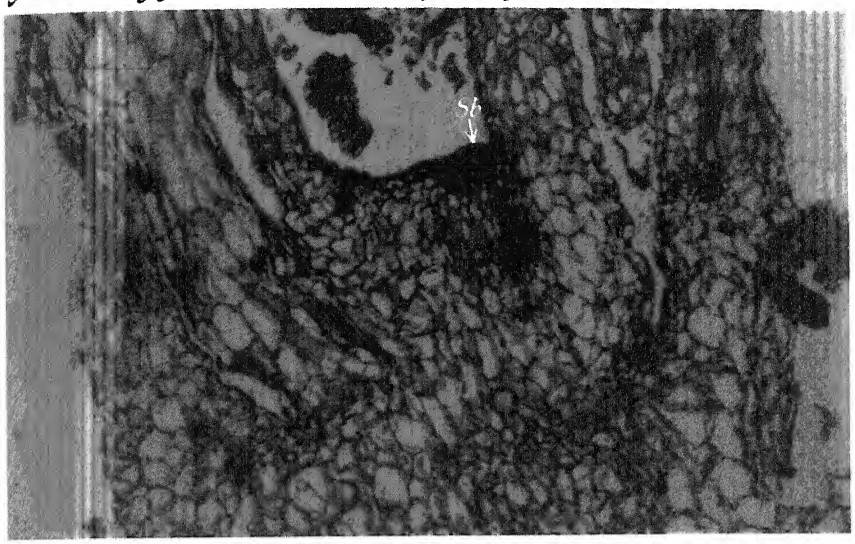


Fig.1 - Shoot Bud Initiation

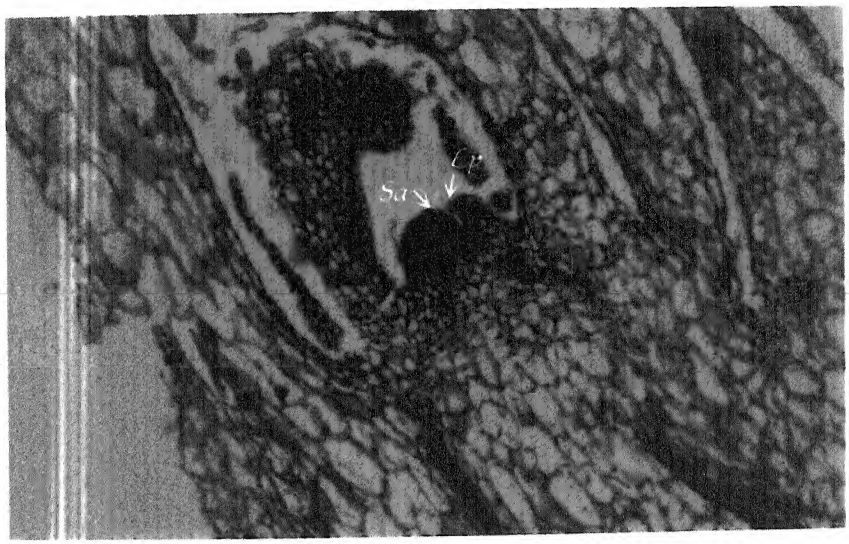


Fig.2 - Shoot Bud Development

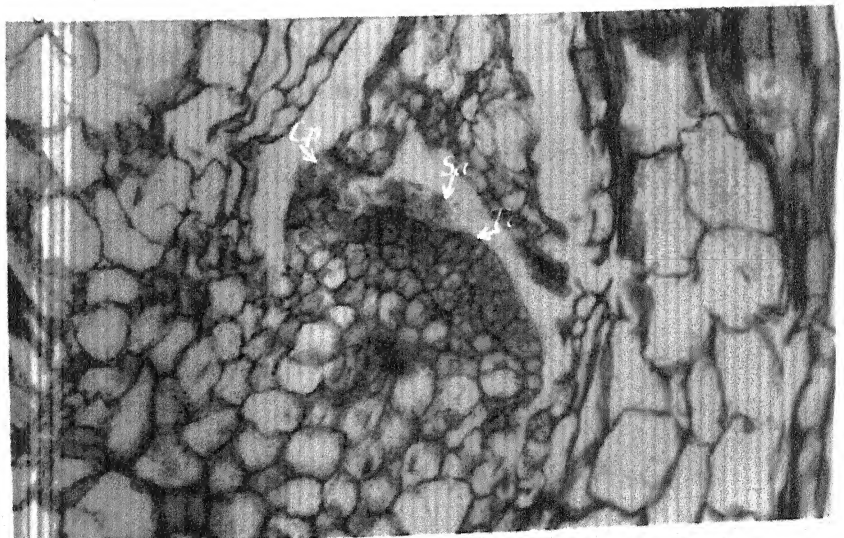


Fig.3 - shoot Bud Exhibiting Tunica-Corpus Organization

Cp - Leaf primordia
Sa - Shoot apex
Sb - shoot bud initiation
Tc - Tunica-Corpus

Plate - 13a

Shoot Bud development in different Genotypes

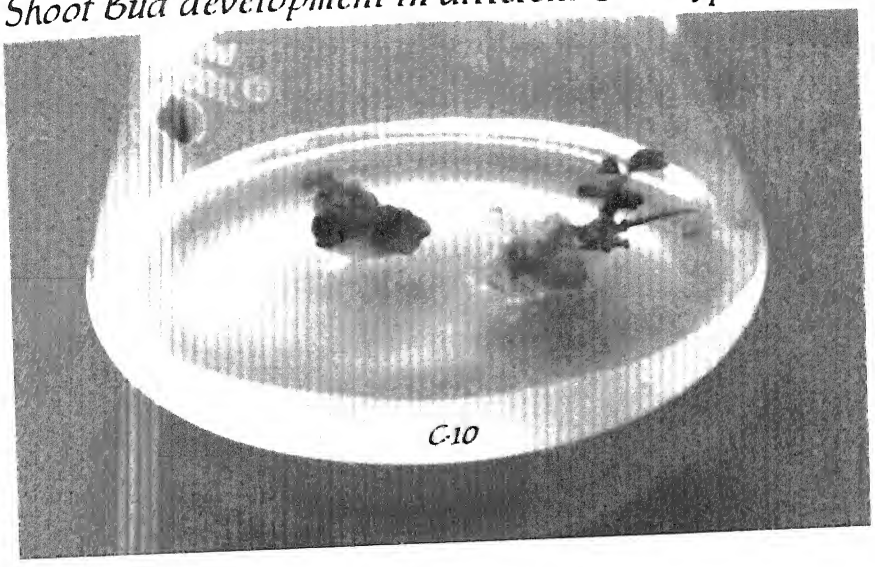


Fig.1 - Hypocotyl

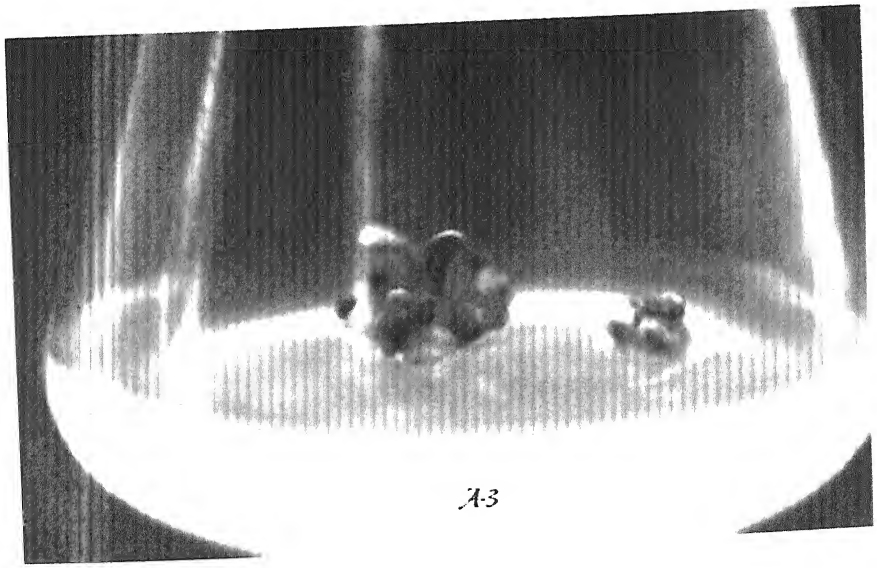


Fig.2 - Cotyledon

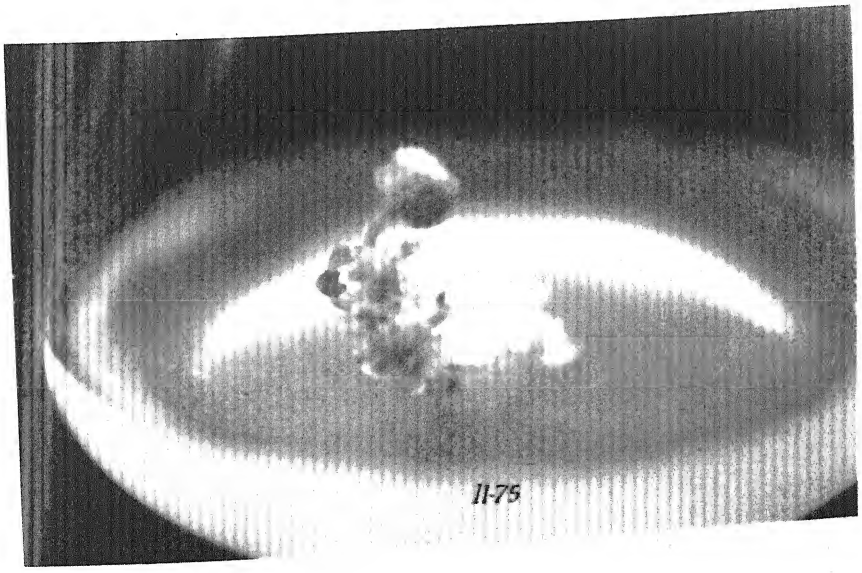


Fig.3 - Epicotyl

Plate - 13b

Fig.4 - Hypocotyl

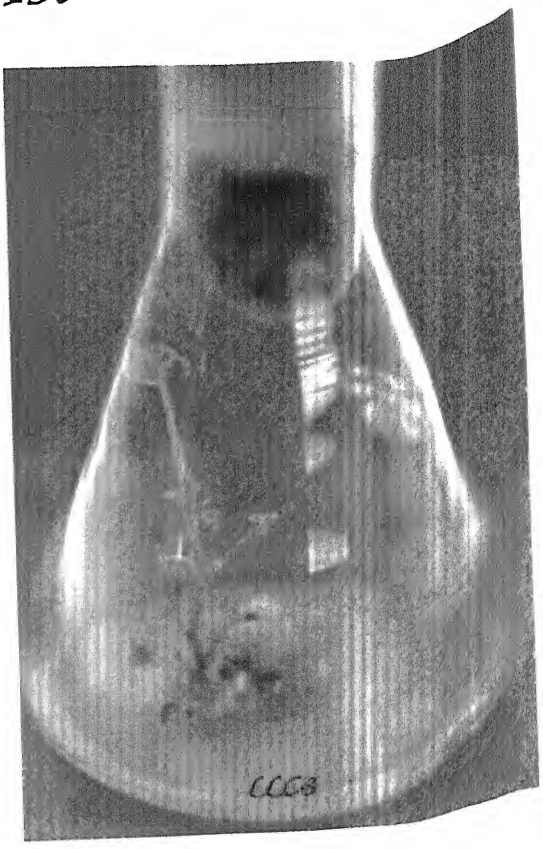


Fig.5 - Cotyledon

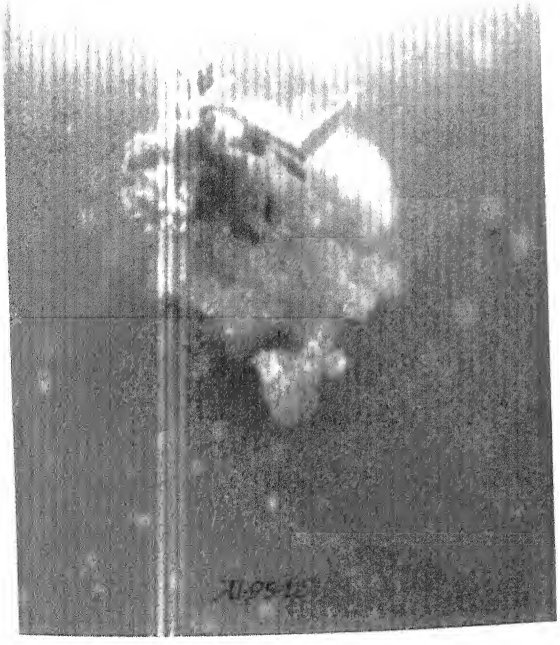


Fig.6 - Hypocotyl

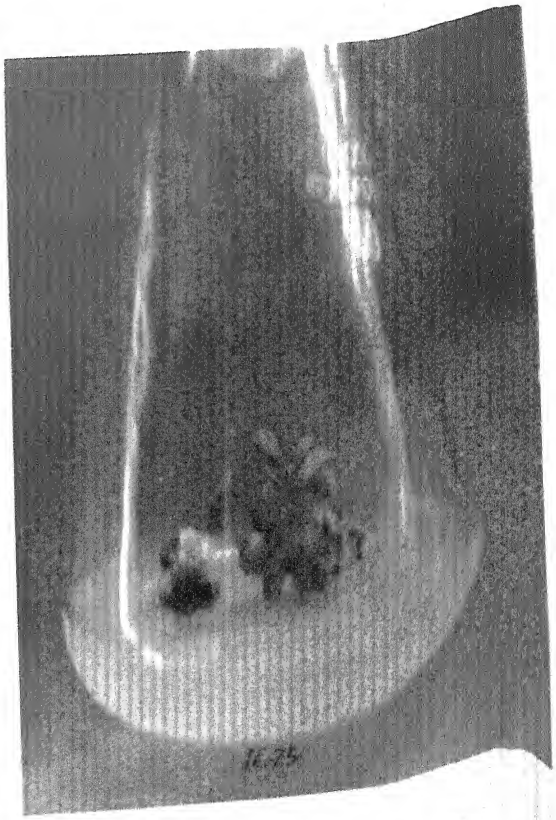


Fig.7 - Hypocotyl

or without 3.0 g/l activated charcoal were formulated. The developing shoots were cultured on these different rooting media and were observed for root induction after 15-20 days of their transfer to these media. While there was no rooting or poor rooting response in the shoots various genotypes under study on the MS salt based rooting media (RM1-RM4), the root development was found better in the shoots grown on RM7 medium (SH+ 0.05 mg/l kinetin+ 2.0 mg/l NAA + 5.0 mg/l IAA) than that occurred on MS medium without hormone and it was limited to the genotypes LLC-3, IL-75 and C-10 only. The best response for root induction and development was observed in RM8 medium (SH+ 0.05 mg/l kinetin+ 2.0 mg/l NAA + 5.0 mg/l IAA +3.0 g/l activated charcoal) in the genotypes LLC-3, IL-75, IG-1212 and C-10. Development of roots was comparatively vigorous and luxuriant in LLC-3 and IL-75 than IG-1212 and C-10 genotypes (Plate-14a & 14b, Fig.1-6). The plantlets of these four genotypes with well developed shoots and roots continued to grow in cultures till the nearly complete exhaustion of culture medium. However, no rooting response could be observed in the genotypes A-3 and AL-95-12 in any of the eight rooting media.

4.1.4.5. Hardening and acclimatization of plantlets

The regenerated plantlets with well developed shoots and roots were taken out of the culture flasks and after washing their roots thoroughly with sterile water they were planted in small plastic pots filled with autoclaved soilrite well soaked with ¼ strength MS basal salt solution containing no growth regulators and sucrose. These pots were covered with polythene begs so as to maintain sufficient moisture inside and maintained in culture conditions of controlled temperature $25^{o}C \pm 2\ ^{o}C$ and 16 hours light/ 8 hours dark photoperiod for 6-7 days. The polythene begs were removed from all the plants after 3 days within this incubation period. The surviving plants were then transferred in the larger pots containing soil, sand and FYM (1:1:1 v/v) and kept in diffused light at room temperature for 8-10 days and subsequently these pots were transferred to the field. After 7-8 days of acclimatization, the plants were transferred to the experimental plot in the field.

The survival of *in vitro* regenerated plants, the somaclones, during hardening and acclimatization has been presented in table-4.15. The plantlets with well developed roots and shoot were obtained in LLC-3, IL-75, C-10 and IG-1212 genotypes only. Out of 78 regenerated plantlets of LLC-3, 60 could survive in the small plastic pots (Plate-15, Fig.1) and out of these 60 plants 52 remained alive in larger pots. These 52 plants were transferred in the experimental plot where 42 plants only could develop to maturity in the field conditions. In case IL-75, out of 30 regenerated plantlets only 12 could survive in the first step of hardening (Plate-15, Fig.2) and none of the regenerated plant could survive in the subsequent stage of hardening and acclimatization of *in vitro* regenerated plants. Whereas, in case of C-10 and IG-1212 genotypes, out of 18 and 11 regenerated plantlets, respectively, none could withstand even the first step of hardening as all the plantlets died within 4-5 days after removing them from cultures and their transfer to soilrite. Hence, all the 42 somaclones developed and grown in the field to maturity belonged to the genotype LLC-3, derived from cotyledon explant.

Table 4.15: Survival of plantlets during hardening process.

Genotype	Number of plantlets transferred to small pots	Number of plants survived in small pots	Number of plants survived in large pots	Number of plants survived in field
LLC-3	78	60	52	42
IL-75	30	12	0	-
C-10	18	0	-	-
IG-1212	11	0	-	-

Plate - 14a

Rhizogenesis in different Genotypes

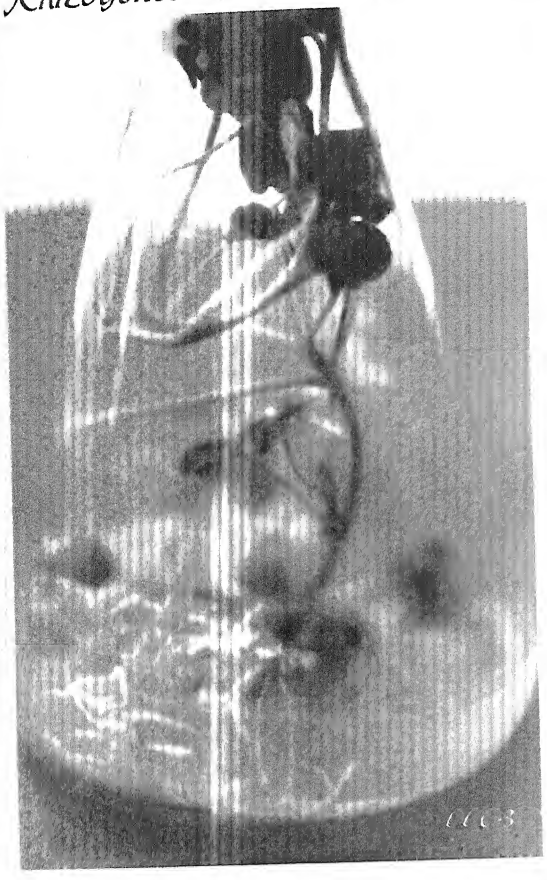


Fig.1 - Cotyledon

Fig.2 - Hypocotyl

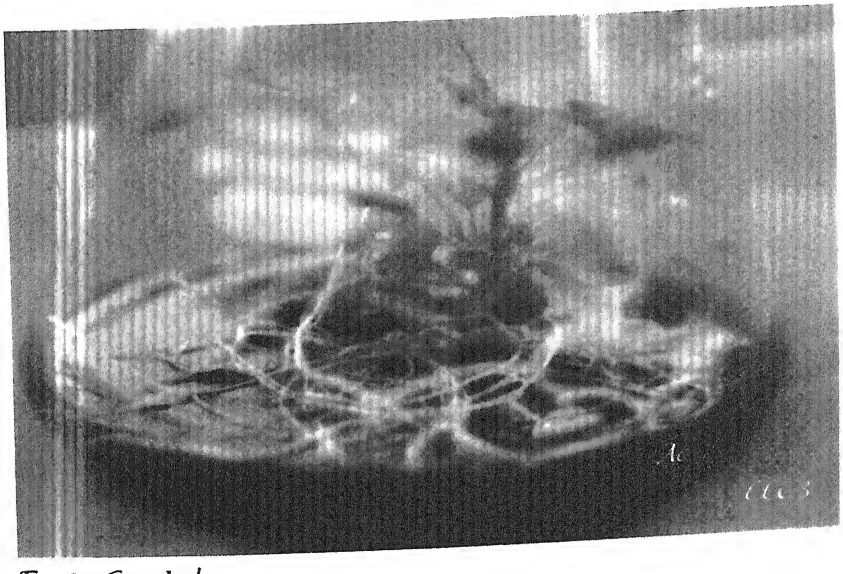


Fig.3 - Cotyledon
Ac - Activated Charcoal

Plate - 14b

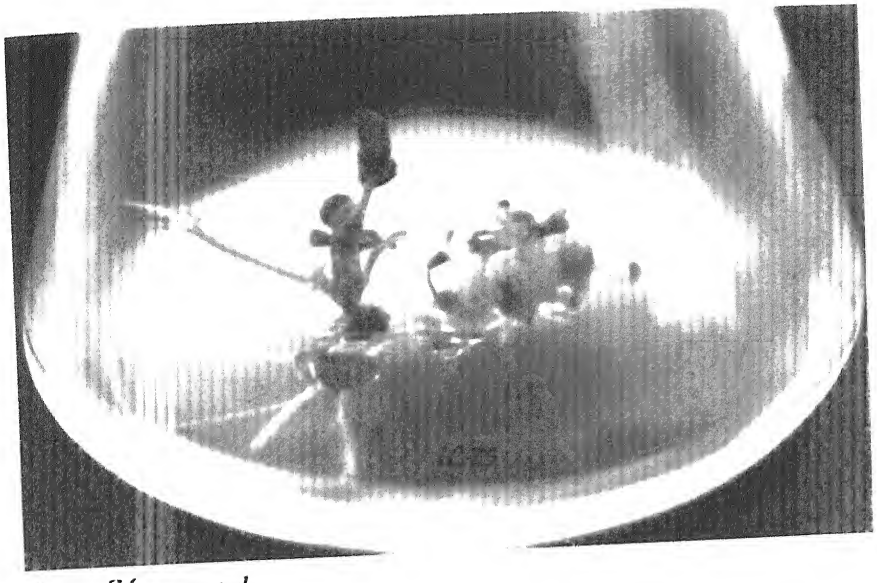


Fig.4 - Hypocotyl

Fig.5 - Hypocotyl

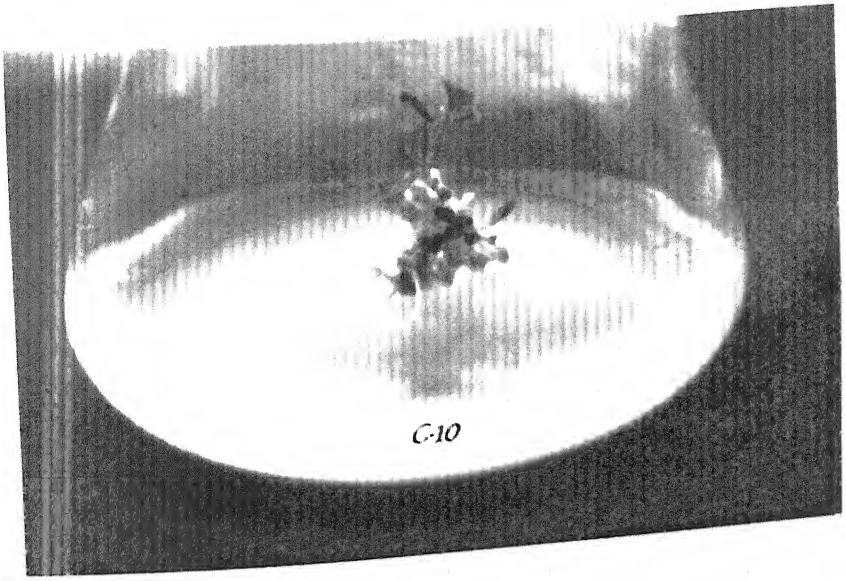


Fig.6 - Hypocotyl

Plate - 15

Hardening of Plantlets

Fig.1 - CCC-3

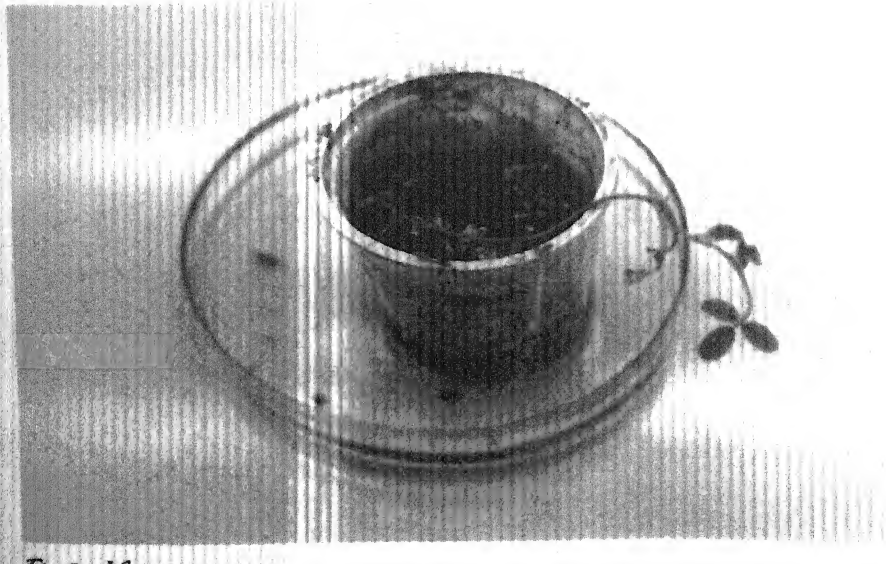


Fig.2 - IC-75

4.2. Evaluation of somaclones and their comparison with the parent material for somaclonal variation.

4.2.1. Morphological traits

The somaclones developed from various explants of different genotypes of lucerne were subjected to hardening followed by their transfer to the field conditions. Among all the somaclones, the plants developed from the callus of cotyledon explant of the genotype LLC-3 only could survive in the field and develop up to maturity (Plate-16, Fig.1 & 2). The performance of these forty-two somaclones for various morphological traits as compared with the mean values of the respective trait of the parent, number of superior and inferior somaclones for the respective trait frequency of somaclonal variation and the frequency distribution of the somaclones for various traits are presented in the table-4.16 and figures 10-21. The regenerated plants exhibited bi-directional variation for all the quantitative traits studied. The mean performance of the somaclones deviated considerably for most of the traits as compared to the parent. Overall performance of somaclones was found better over to that of the parent with respect to the number of nodes and internodes, stem girth and flower size.

The maximum number of superior somaclones recorded was for stem girth (34) followed by the number of superior somaclones for number of nodes (26) and length of internodes (20). The minimum number of superior somaclones over the parent was for per cent dry matter content (2) followed by fresh leaf weight and flower length (3, each).

The maximum number of somaclones which exhibited data lower than that of the parent was for dry stem weight (22) followed by the number of somaclones for dry weight of leaf (17) and leaf/stem weight ratio on fresh weight basis (16). None of the somaclones was found inferior to the parent for the traits such as, number of nodes and stem girth.

Plate - 16

Somaclones in Field

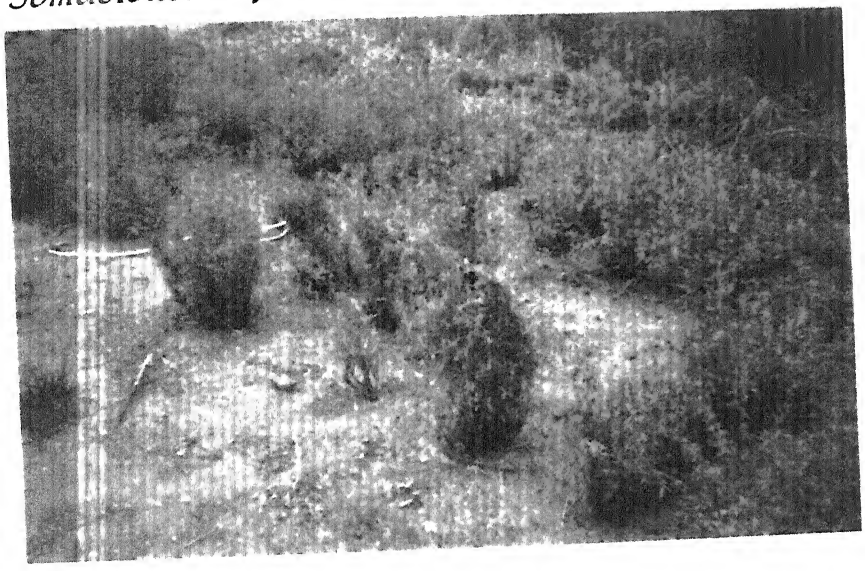


Fig.1 - Somaclones

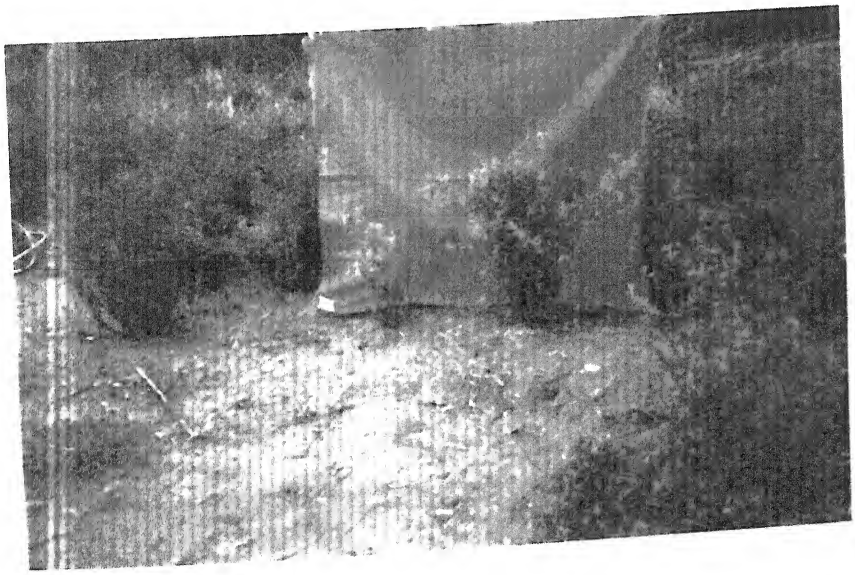


Fig.2 - Somaclones

Table 4.16 – Somaclonal variation for morphological traits in LLC-3 genotype of *M.sativa*

Sl no.	Character	Parent mean	Somaclones			Number of variants		Somaclonal variation frequency (%)
			Mean	Minimum	Maximum	Superior to parent	Inferior to parent	
1	Plant height	103.60	103.12	63.00	144.52	11.00	13.00	54.76
2	Branches/plant	8.40	9.93	1.00	15.00	6.00	5.00	26.19
3	No. of nodes	10.60	14.86	10.00	21.00	26.00	0.00	61.90
4	Length of internodes	4.94	5.55	3.42	7.47	20.00	9.00	69.05
5	Stem girth	1.13	1.32	1.12	1.52	34.00	0.00	80.95
6	Leaf weight (Fresh)	80.78	72.26	10.00	162.29	3.00	7.00	23.81
7	Stem weight (Fresh)	158.24	138.02	20.00	306.67	8.00	15.00	54.76
8	Leaf/stem weight ratio	0.51	0.52	0.50	0.53	12.00	16.00	66.67
9	Total green weight	239.02	210.28	30.00	468.96	5.00	14.00	45.24
10	Dry leaf weight	16.34	15.90	2.00	55.30	7.00	17.00	57.14
11	Dry stem weight	33.92	30.86	3.20	79.08	12.00	22.00	80.95
12	Total dry weight	50.26	46.76	5.20	134.38	5.00	14.00	45.24
13	Dry matter content (%)	21.03	22.24	17.33	28.65	2.00	3.00	11.90
14	Flower length	1.09	1.03	0.93	1.32	3.00	4.00	16.67
15	Flower width	0.56	0.68	0.50	0.88	17.00	2.00	45.24
16	Flower size	0.61	0.70	0.47	1.16	5.00	4.00	21.43

The range of bi-directional variation was substantial in case of plant height, branches per plant, leaf and stem weight (fresh and dry), total green weight, total dry weight, dry matter content and flower size.

Frequency of variation in the regenerated plants varied considerably for various morphological characters as indicated by the somaclonal variation frequency. While for stem girth and dry weight stem most of the somaclones (80.95 %) deviated significantly from the parent, minimum variation was noticed with regard to per cent dry matter content (11.90 %). Very high frequency of somaclonal variation was also noted in case length of internode (69.05 %), leaf to stem ratio of fresh weight (66.67 %) and the number of nodes (61.9 %). Moderate incidence of variation was seen for dry weight of leaf (57.14 %), plant height (54.76 %), total green and dry weight (45.24 %, each), fresh stem weight (54.76 %), flower width (47.62 %). In case of other traits such as branches per plant, fresh leaf weight, flower length, flower size and per cent dry matter content etc. low incidence of variation was observed.

The frequency distribution of somaclones for various morphological traits, as per the variation exhibited on both the directions in terms of the mean values of the parent and the similarity exhibited for some of the somaclones with the parent are described.

1. Plant height

Increased bi-directional variation for plant height was observed in somaclones as evident from the frequency distribution of plant height in somaclones (Fig.10). As compared to the mean parental plant height of 103.60 cm., lowest plant heights (63-74.65 cm.) were recorded in 9 somaclones and the highest (132.88-144.52 cm.) were recorded in 3 somaclones. However seven somaclones performed similar to parent (97.9-109.58 cm.) for plant height.

2. Branches per plant

The frequency distribution of branches per plant in somaclones indicated increased bi-directional variation for branches per plant (Fig.11). Lowest number of branches

(1-3) was exhibited by in one somaclone and the highest number of branches (13-15) was recorded in three somaclones as compared to the number of branches in parent plants (8-9). Nevertheless, 13 somaclones were similar to parent (7-10) for branches per plant.

3. Number of nodes per branch

Increased bi-directional variation for number of nodes was found in somaclones from the frequency distribution of number of nodes per branch (Fig.12), though the variation towards lower number of nodes was negligible. Minimum number of nodes (10-12) was observed in three somaclones and maximum number of nodes (19-21) was found in one somaclone as compared to the parent (10-11 nodes). 13 somaclones had the same number of nodes per branch as the parent (10-11 nodes).

4. Length of internodes

Mean Internodal length of somaclones showed increased variation in both lower and higher distribution of the parental mean as revealed by the frequency distribution of internodal length (Fig.13). As compared to the parental mean internodal length (4.94 cm.), minimum length (3.42-4.10 cm.) was observed in 9 somaclones and maximum length (7.47 cm.) was observed in 2 somaclones while 6 somaclones were similar to parent (4.77 cm.). Maximum number of somaclones (more than 9) were observed to possess an internodal length of 6.80 to 7.47 cm., much higher than that of the parent.

5. Stem girth

While the parental mean stem girth was 1.13 cm., most the somaclones exhibited higher stem girth, falling between 1.12 and 1.52 cm. (Fig.14). A maximum of 12 plants exhibited stem girth of 1.24-1.29 cm. In general the frequency of plants exhibiting increased stem decreased with increase in stem girth.

6. Fresh leaf weight

Variation for Fresh leaf weight in somaclones also increased bi-directional as indicated by the frequency distribution (Fig.15). Lowest fresh leaf weight (10.00-

31.76 g.) was recorded in 7 somaclones whereas highest value (162.29 g.) was recorded in only one somaclone. Seven somaclones behaved similar to parent (75.27-97.02 g.). 12 somaclones were found to fall between 31.76 and 53.51 g., which was the maximum frequency in any class.

7. Fresh stem weight

Increased bi-directional variation for fresh stem weight was observed in somaclones from the frequency distribution of fresh stem weight (Fig.16) with more somaclones performing poorer than parent. Minimum fresh stem weights (20.00-60.95 g.) were recorded in 15 somaclones and maximum fresh stem weight in the range (265.71-306.67g.) was recorded in one somaclone only. Four somaclones were occurring in the proximal range (101.91-142.869 g.) of parental mean fresh stem weight.

8. Fresh leaf/ stem weight ratio

A somewhat bi-directional variation for fresh leaf/ stem ratio was also observed in somaclones as evident from the frequency distribution of fresh leaf/ stem ratio. Lowest leaf/stem ratios (0.37-0.57) were recorded in 16 somaclones and high ratio was recorded only in one somaclone, as compared to parental leaf stem /ratio weight ratio, while 10 somaclones were similar to parent (0.53-0.70) with respect to leaf to stem fresh weight ratio.

9. Total green weight

The frequency distribution of total green weight in the somaclones of LLC-3 genotype indicated enhanced variation in both higher and lower directions as compared to parent (Fig.17). A maximum 14 somaclones showed poorest performance in terms of total green weight (30-92.7 g.) and only one somaclone exhibited the highest total green weight (468.96g). Most of the remainingsomaclones had distribution around the parent in the range of 155.42 to 280.83 g. More number of somaclones exhibited lower total green weight in the frequency distribution.

10. Dry leaf weight

Increased bi-directional variation for leaf dry weight was observed in somaclones as evident from the frequency distribution (Fig.18) with more somaclones recording lower dry leaf weights, as compared to the mean parental dry leaf weight of 16.34 g. The lowest dry weights (2.00-9.62g.) were recorded in 17 somaclones and highest (32.46-55.30 g.) were recorded in one somaclone each. Nine somaclones responded similar to parent (9.62-17.23 g.) for dry leaf weight.

11. Dry stem weight

Increased bi-directional variation on a pattern similar to fresh stem weight was observed in somaclones as evident from the frequency distribution of stem dry weight in these somaclones (Fig.19). Minimum dry stem weights (3.20-14.04 g.) were recorded in 16 somaclones while maximum dry stem weights (68.24-79.08 g.) were recorded in 2 somaclones. Six somaclones (24.88-35.72 g.) were similar to parent (33.92 g.) for dry stem weight.

12. Total dry weight

The increase in the variation for dry matter weight among the somaclones was on a similar pattern for the trait as compared to total green weight. While very few somaclones behaved similar to parent, most of them had a lower dry matter weight with 14 somaclones performing extremely poor (5.20-20.11g.). Two somaclones recorded dry matter yield higher than that of the parent. Six somaclones fell in the 79.75-94.66g ranges while five somaclones yielded 94.66 to 134.38 g dry matter.

13. Dry matter content

A near normal distribution of dry matter content was observed in somaclones, indicating increased bi-directional variation. Lowest dry matter content (17.33 %) was recorded in one somaclone and highest (26.86-28.65 %) were recorded in 2 somaclones as compared to mean parental dry matter content of 21.03 %. Eight somaclones exhibited (24.58%) dry matter content and 11 somaclones recorded 22.3% dry matter content, which were quite closer to the mean value of the parent.

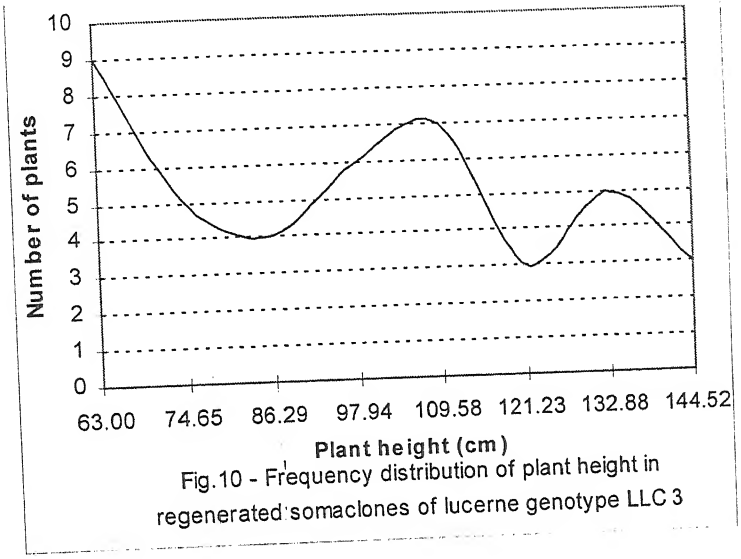


Fig.10 - Frequency distribution of plant height in regenerated somaclones of lucerne genotype LLC 3

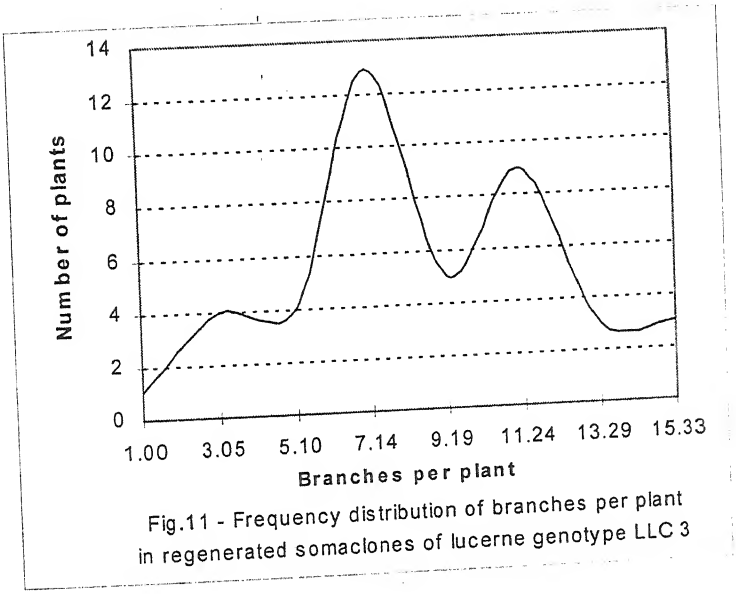


Fig.11 - Frequency distribution of branches per plant in regenerated somaclones of lucerne genotype LLC 3

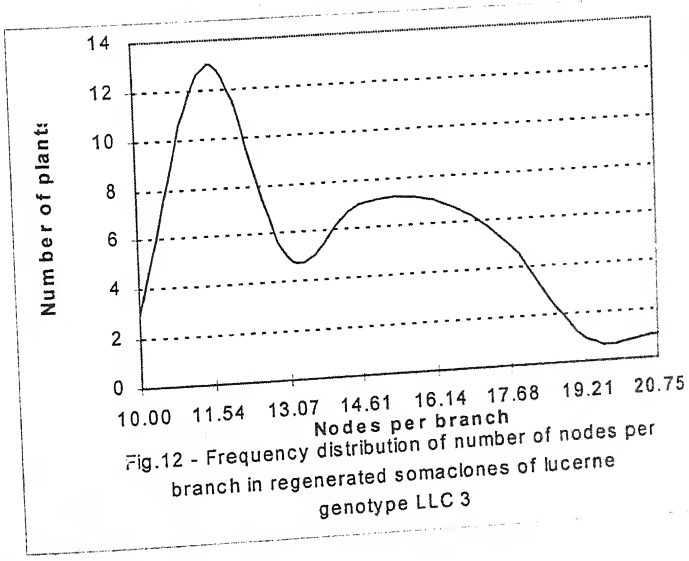


Fig.12 - Frequency distribution of number of nodes per branch in regenerated somaclones of lucerne genotype LLC 3

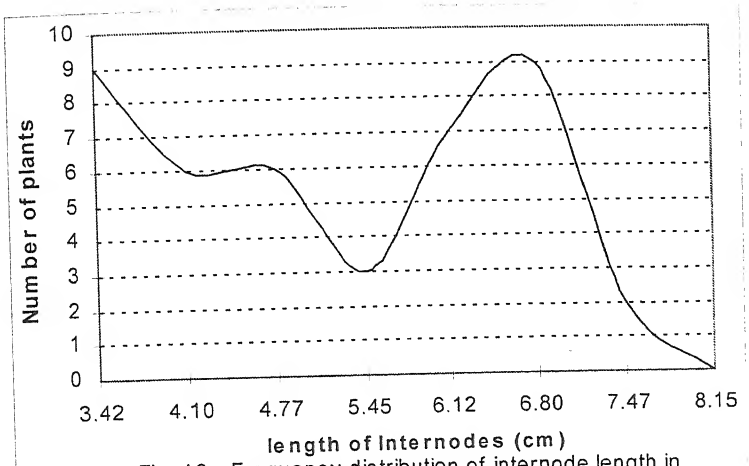


Fig. 13 - Frequency distribution of internode length in regenerated somaclones of lucerne genotype LLC 3

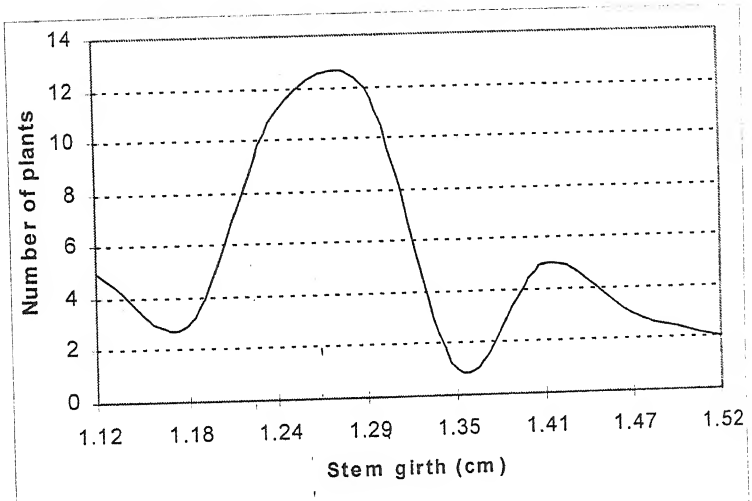


Fig.14 - Frequency distribution of stem girth in regenerated somaclones of lucerne genotype LLC 3

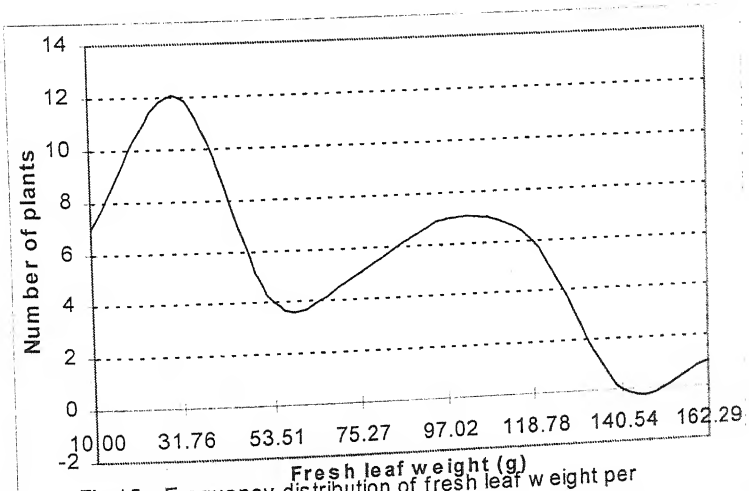


Fig.15 - Frequency distribution of fresh leaf weight per plant in regenerated somaclones of lucerne genotype LLC 3

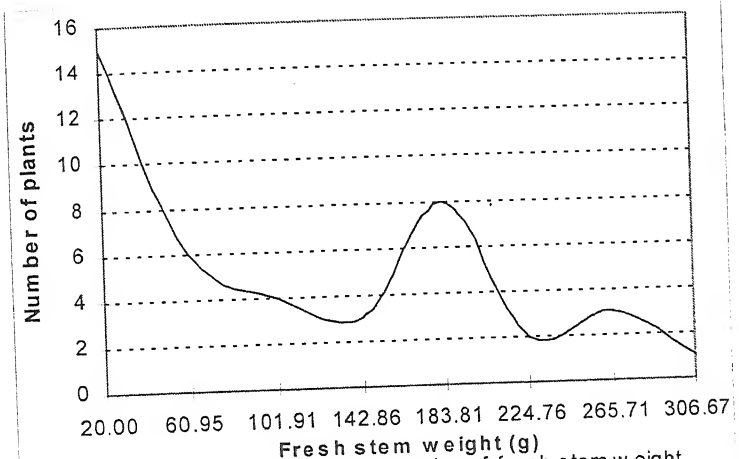


Fig.16 - Frequency distribution of fresh stem w eight per plant in regenerated somaclones of lucerne genotype LLC 3

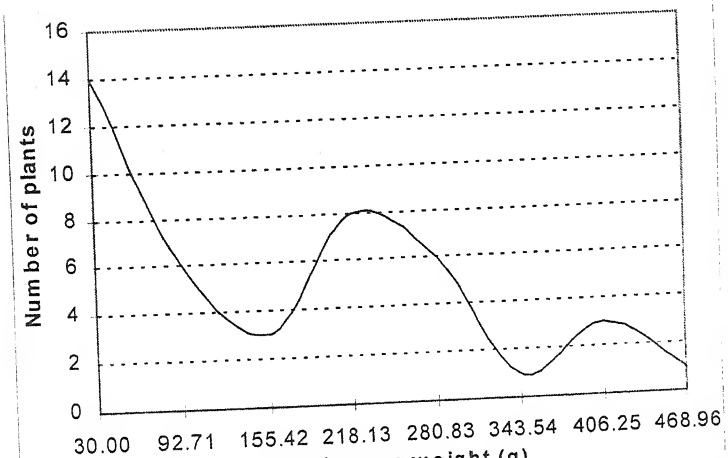


Fig.17 - Frequency distribution of total green w eight per plant in regenerated somaclones of lucerne genotype LLC 3

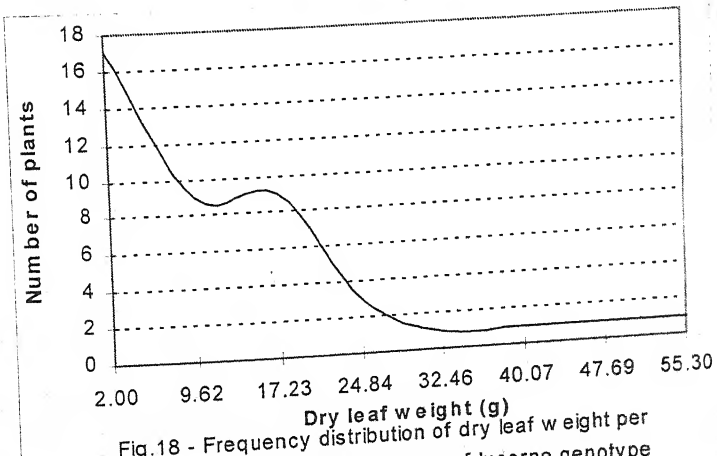


Fig.18 - Frequency distribution of dry leaf w eight per plant in regenerated somaclones of lucerne genotype LLC 3

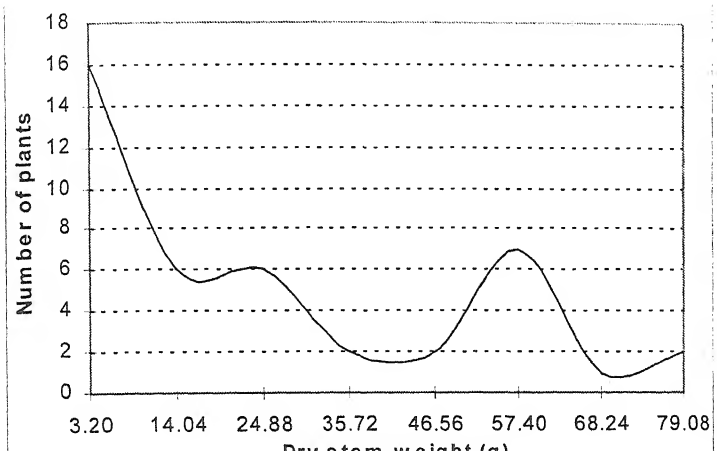


Fig.19 - Frequency distribution of dry stem weight per plant in regenerated somaclones of lucerne genotype LLC 3

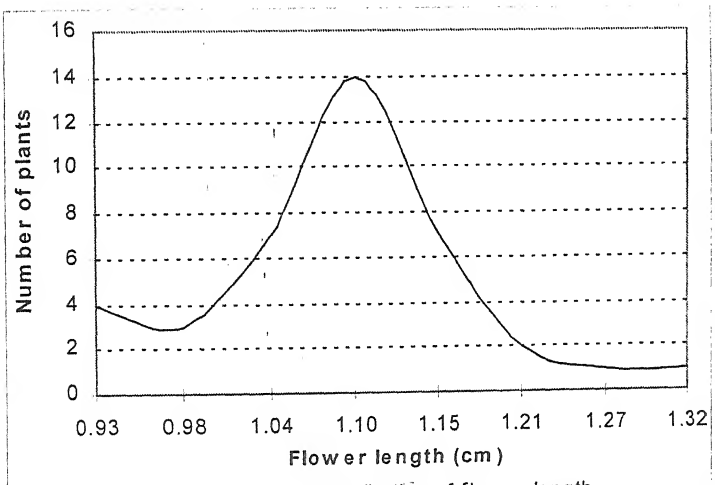


Fig.20 - Frequency distribution of flower length in regenerated somaclones of lucerne genotype LLC3

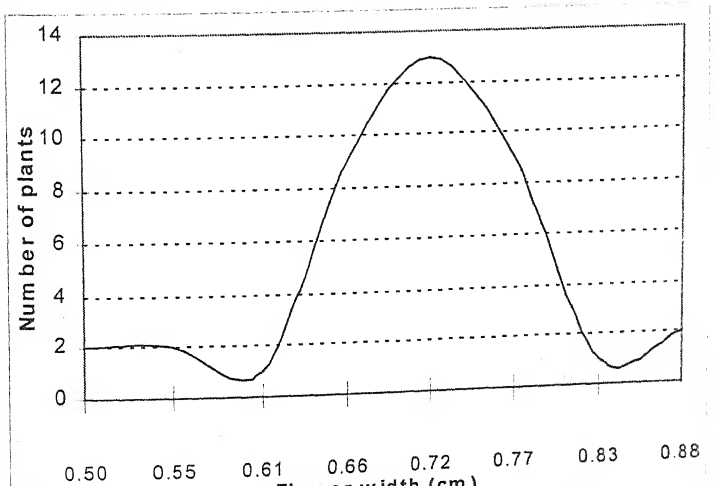


Fig.21 - Frequency distribution of flower width in regenerated somaclones of lucerne genotype LLC

14. Flower length

The distribution for flower length in the somaclones followed a normal distribution with most (14) somaclones following around the parental mean (1.09 cm.). Increased variation for flower length was observed in the somaclones as evident for the frequency distribution of flower length (Fig.20). Minimum flower length (0.93-0.98 cm.) was observed in 4 somaclones and maximum flower length (1.27-1.32 cm.) was observed in one somaclone as compared to parental mean.

15. Flower width

This trait also followed an apparent normal distribution, but with greater proportion of somaclones showing higher flower width than the parent. Increased variation for flower width was however observed in somaclones (Fig.21). Minimum flower width (0.50 cm.) was observed in 2 somaclones and maximum flower width (0.83-0.88 cm.) was also recorded in 2 somaclones as compared to parental mean of flower width (0.56 cm.). Only 2 somaclones were similar to parent (0.55-0.61 cm.).

16. Flower size

The size of flower in somaclones exhibited a unimodal distribution besides the bi-directional increase in variation with respect to mean parent value. Though 12 somaclones recorded flower size between 0.75 and 0.84 square cm., the higher (1.09-1.16 sq.cm.) and the lower (0.50-0.58 sq.cm.) ranges were recorded by one and four somaclones respectively. Most of the somaclones exceeded the parental flower size of 0.61 square cm.

4.2.2. Biochemical characterization of somaclones

Isozymes of phophoglucoisomerase (PGI), phosphoglucomutase (PGM), Glucose 6-phosphatedehydrogenase (G6PDH), Aspartate aminotransferase (AAT), Superoxide dismutase (SOD) and Esterase of 6 somaclones and their parent were analysed using PAGE (Polyacrylamide gel electrophoresis). The results indicated that these

somaclones differed among themselves and with their parent for isozymes of PGI, PGM, G6PDH, AAT, SOD and Esterase (Table-4.17; Fig.22).

Table 4.17 : Zymogram analysis of somaclones and the parent

Parent plant LLC-3	**PGI**	**PGM**	**G6PDH**	**AAT**	**SOD**	**Esterase**
Number of somaclones analyzed	6	6	6	6	6	6
Total number Of bands	2	3	4	3	4	4
Total number of bands in the parent	2	2	2	3	2	4
Number of somaclones differing from the parent	1	6	3	6	3	4

Phophoglucoisomerase (PGI)-

PGI contained single loci with a maximum of three alleles exhibiting polymorphism among somaclones. Somaclone number 3, 4 and 6 were confirming to parent with dimorphic bands whereas somaclone number 5 was monomorphic and somaclone number 1and 2 were trimorphic with a heterodimer indicating their heterozygous nature (Plate-17a, Fig.1; Fig - 22A).

Phosphoglucomutase (PGM)

PGM showed 3 loci with a maximum of 2 alleles while parent exhibited monomorphism for all the 3 loci, six somaclones were dimorphic for PGM-1 and all the somaclones confirmed to parent for PGM-2 and PGM-3 loci. Thus, somaclones exhibited heterozygosity for PGM1 whereas somaclone number 5 did not contain any bands in PGM1 (Plate-17a, Fig2; Fig. - 22B).

Glucose 6 phosphatedehydrogenase (G6PDH)

G6PDH contained 2 loci with a maximum of 2 alleles. In G6PDH-1, somaclone number 3 and 4 were dimorphic whereas other somaclones and the parent did not contain any bands. In G6PDH-2, somaclones numbers 1, 2, 3, 4 and 6 were confirming to parent with dimorphic nature whereas, bands were not observed in somaclone number 5 (Plate-17a, Fig.3; Fig. – 22C).

Aspartate aminotransferase (AAT)

AAT contained 2 loci with a maximum of 2 alleles. In AAT-1, somaclone numbers 2, 3, 4, 5 and 6 were confirming to parent with monomorphic band whereas in somaclone number 1, bands were not present. In AAT-2, both the parent and somaclone number 1 were dimorphic and somaclone number 2, 3, 4, 5 and 6 were monomorphic in nature (Plate-17b, Fig.4; Fig. - 22D).

Superoxide dismutase (SOD)

SOD exhibited two loci wherein SOD-1 was monomorphic both in parent and the somaclones, SOD-2 was monomorphic in parent and in somaclone number 1, 2 and

Plate - 17a

Isozyme Polymorphism

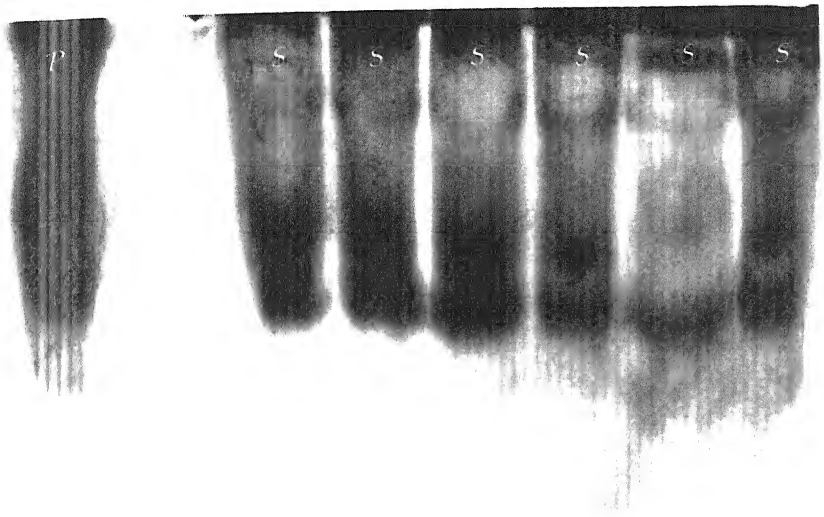


Fig.1 - Phosphoglucoisomerase

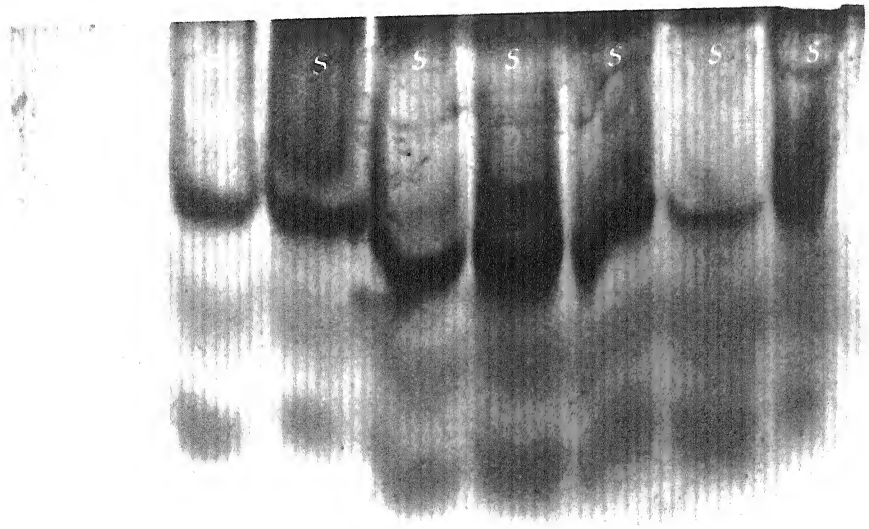


Fig.2 - Phosphoglucomutase

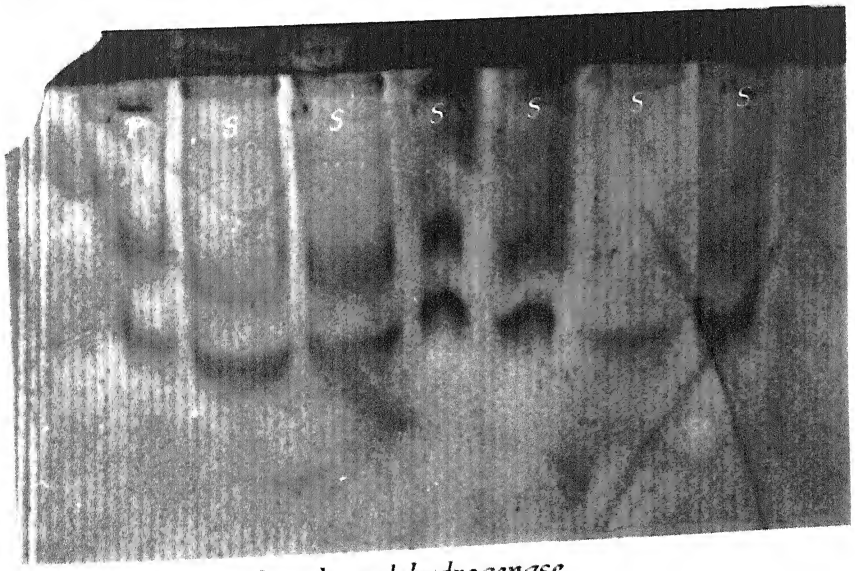


Fig.3 - Glucose 6-phosphate dehydrogenase

Plate - 17b

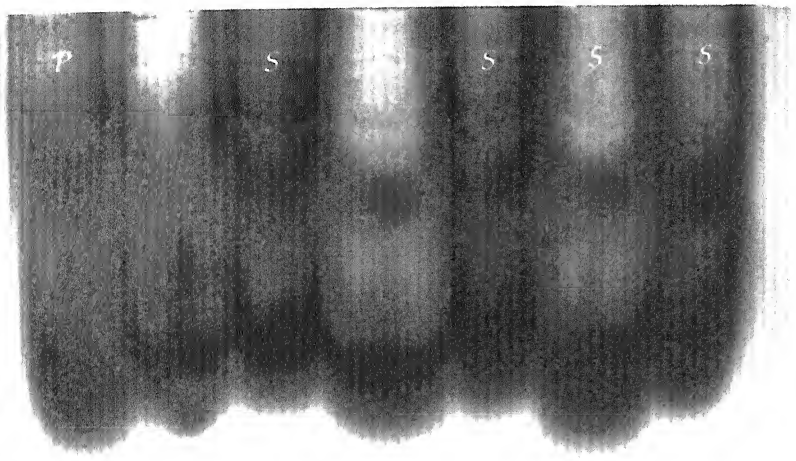


Fig.4 - Aspartate amino transferase

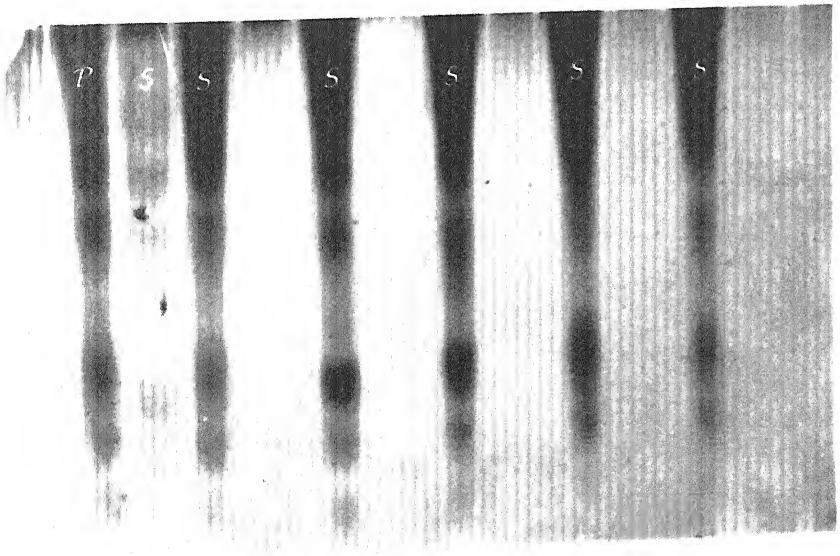


Fig.5 - Esterase
P - Parent
S - Somaclone

Fig. 22 - Zymogram of various somaclones and the parent

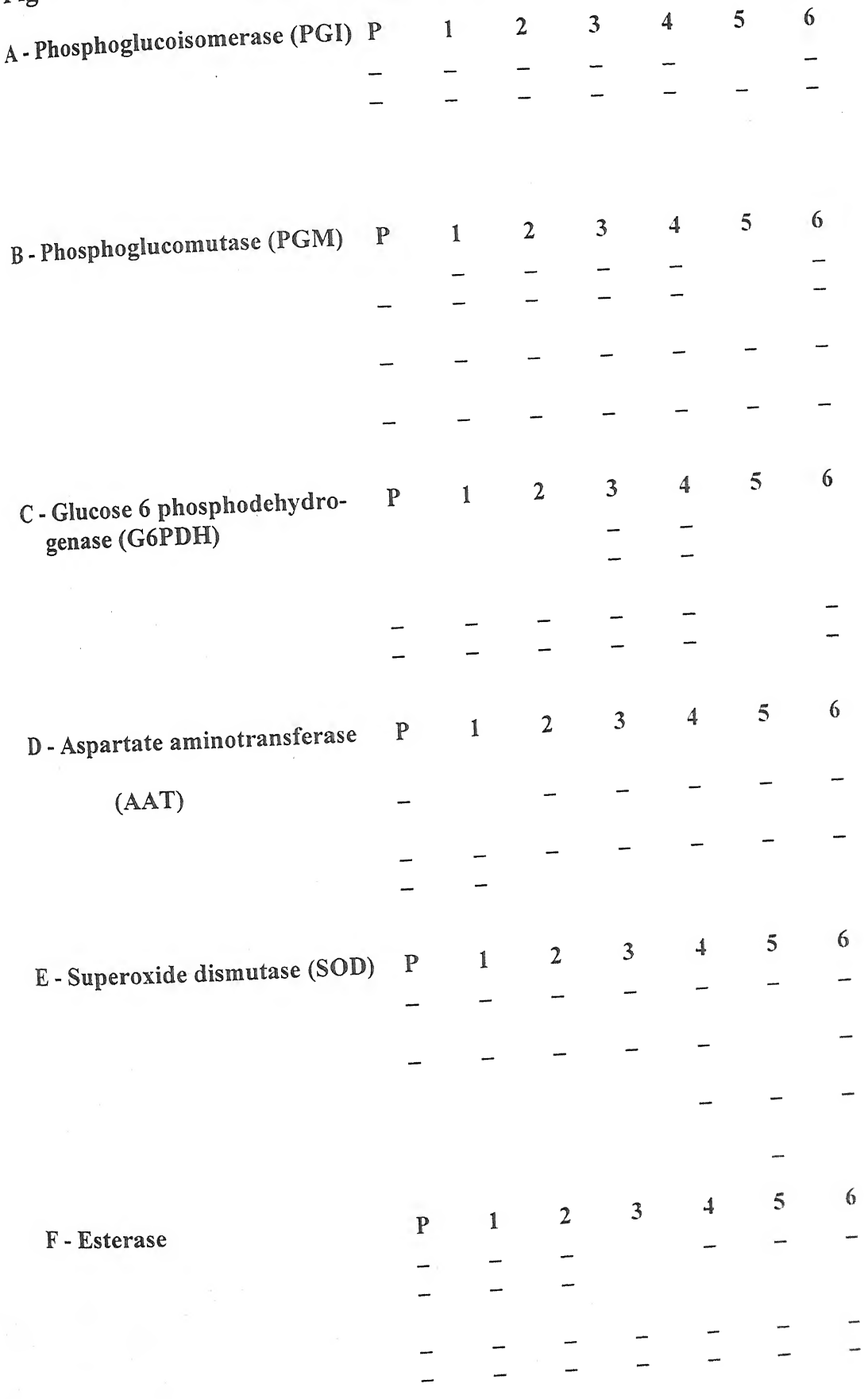
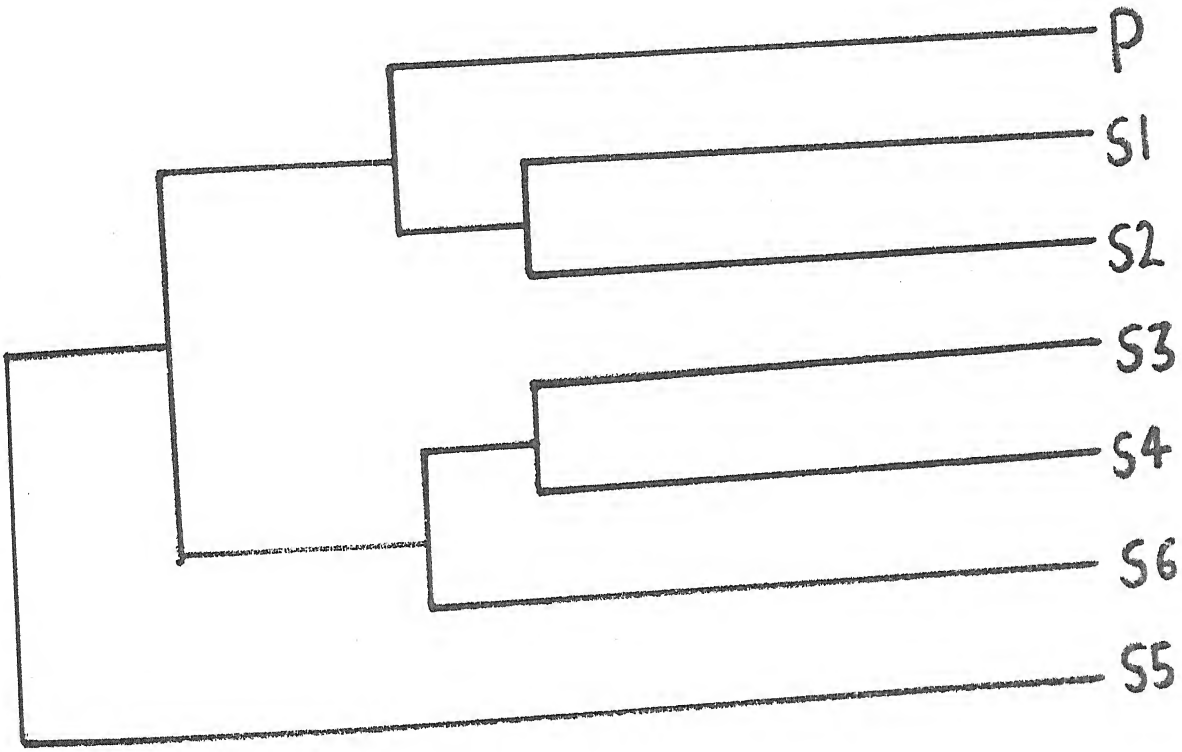


Fig. 23 – Dendrogram analysis based on isozyme banding pattern



3 and the somalone numbers 4, 5 and 6 were dimorphic. Thus somaclones 4, 5 and 6 were heterozygous in comparison to rest of the somaclones (Fig. – 22E).

Esterase (Est)

Esterase contained 2 loci with a maximum of 2 alleles. In Est-1, somaclone number 1 and 2 were confirming to parent with dimorphic bands and somaclone numbers 4, 5 and 6 were monomorphic whereas somaclone number 3 did not contain any band. In Est-2, both parent and somaclones were dimorphic and showed no variation among themselves (Plate-17b, Fig.5; Fig. – 22F).

Dendrogram analysis based on isozyme banding pattern

The dendrogram analysis using data from six isozymes in lucerne somaclones S1 and S2 were found genetically closer to the parent than somaclone S5. Further, there were three major groups of somaclones *viz*. somaclones S1 and S2 in the first group, somaclones S3, S4, and S6 in second group while somaclone S5 was found distantly related to parent. This study confirmed the frequency of somaclonal variation induced *in vitro* (Fig.-23).

4.2.3. RAPD polymorphism

RAPD profiles were analysed among somaclones and their parent for 6 primers. Polymorphism was observed for all the primers used in this study. This further confirmed the somaclonal nature of the regenerants (Table 4.18; Fig.-24). The nature of the polymorphism obtained for each primer is presented here, below:

OPJ-01

Totally eleven alleles were observed among five somaclones and their parent. While two somaclones (*viz* S1 and S4) shared a similar phenotype which was different than parent, S5 was similar to parent. Somaclone S2 exhibited a unique allele which was uncommon and thus S2 was the only somaclone that exhibited all the allele (Plate-18, Fig.1; Fig. - 24A).

Table 4.18 : RAPD analysis of somaclones and the parent

Parent plant LLC-3	**OPJ-01**	**OPJ-04**	**OPJ-05**	**OPJ-06**	**OPM-04**	**OPE-01**
Number of somaclones analyzed	5	5	5	5	5	5
Total number of bands	11	10	8	9	8	11
Total number of bands in the parent	8	9	6	8	7	4
Number of somaclones differing from the parent	4	4	5	5	4	5

OPJ-04

Ten alleles were scored in the parent and none of the somaclones shared their phenotype with the parent. S1and S2 somaclone shared a common phenotype. Thus, there were five different phenotypes observed among somaclones and their parent (Plate-18, Fig.2; Fig. - 24B).

OPJ-05

Totally 8 alleles were scored among somaclones and their parent. Somaclones S1 and S4 shared a common phenotype while the parent did not share phenotype with any of the somaclones. Somaclones S2 and S3 were very distinct than the rest of the somaclones (Plate-18, Fig.3; Fig. – 24C).

OPJ-06

In all, nine alleles were scored which were observed in the parent. Four different phenotypes were observed out of which only two somaclones (S3 and S4) shared a common phenotype. None of the somaclones were similar to parent (Plate-18, Fig.4; Fig. – 24D)

OPM-04

As observed in case of OPJ-01, polymorphism was not observed between S5 and the parent. Totally 8 alleles were scored which was observed in somaclone S2. None of the other somaclones were similar to each other (Plate-18, Fig.5; Fig. – 24E).

OPE-01

Maxomum polymorphism was observed for this primer between somaclones their parent. While a maximum of 11 alleles were scored in few somaclones (S3, S4 and S5), which shared a common phenotypes, parent was very distinct in its phenotype. Totally 4 different phenotypes were observed. The parent showed only 4 alleles (Plate-18, Fig.6; Fig. – 24F).

Plate - 18

DNA Polymorphism with Different Primers

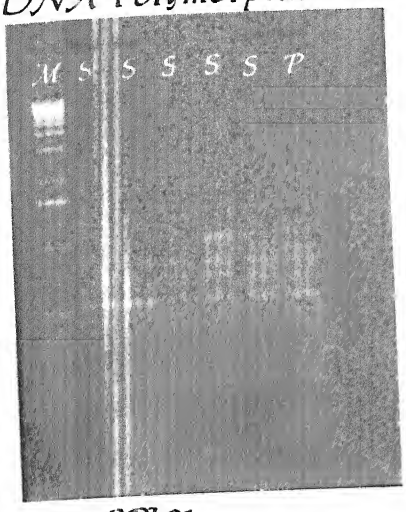


Fig.1 - OPJ-01

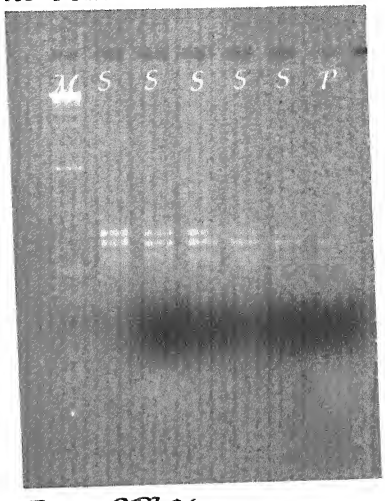


Fig.2 - OPJ-04

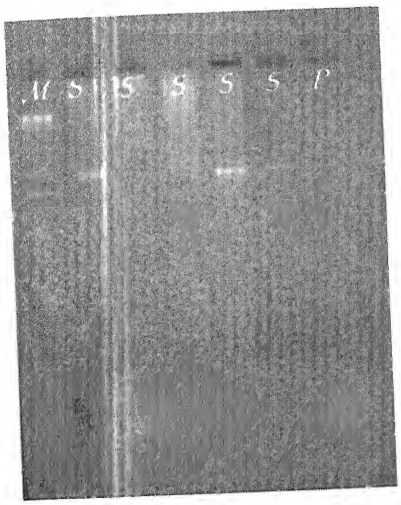


Fig.3 - OPJ-05

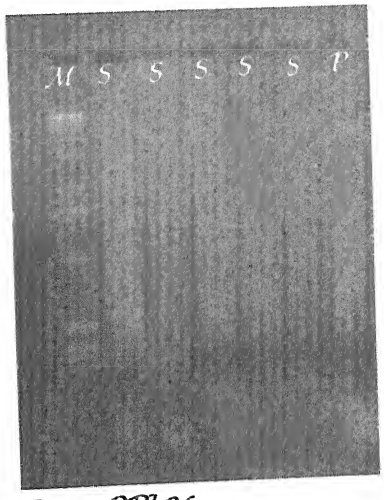


Fig.4 - OPJ-06

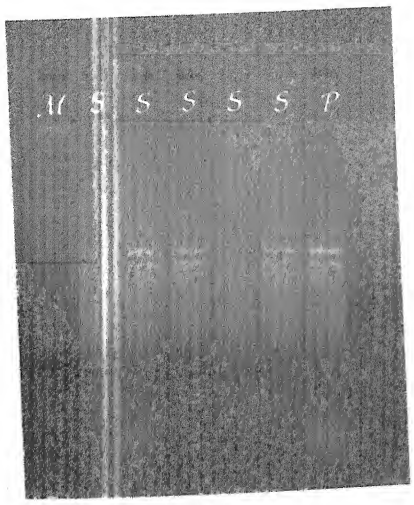
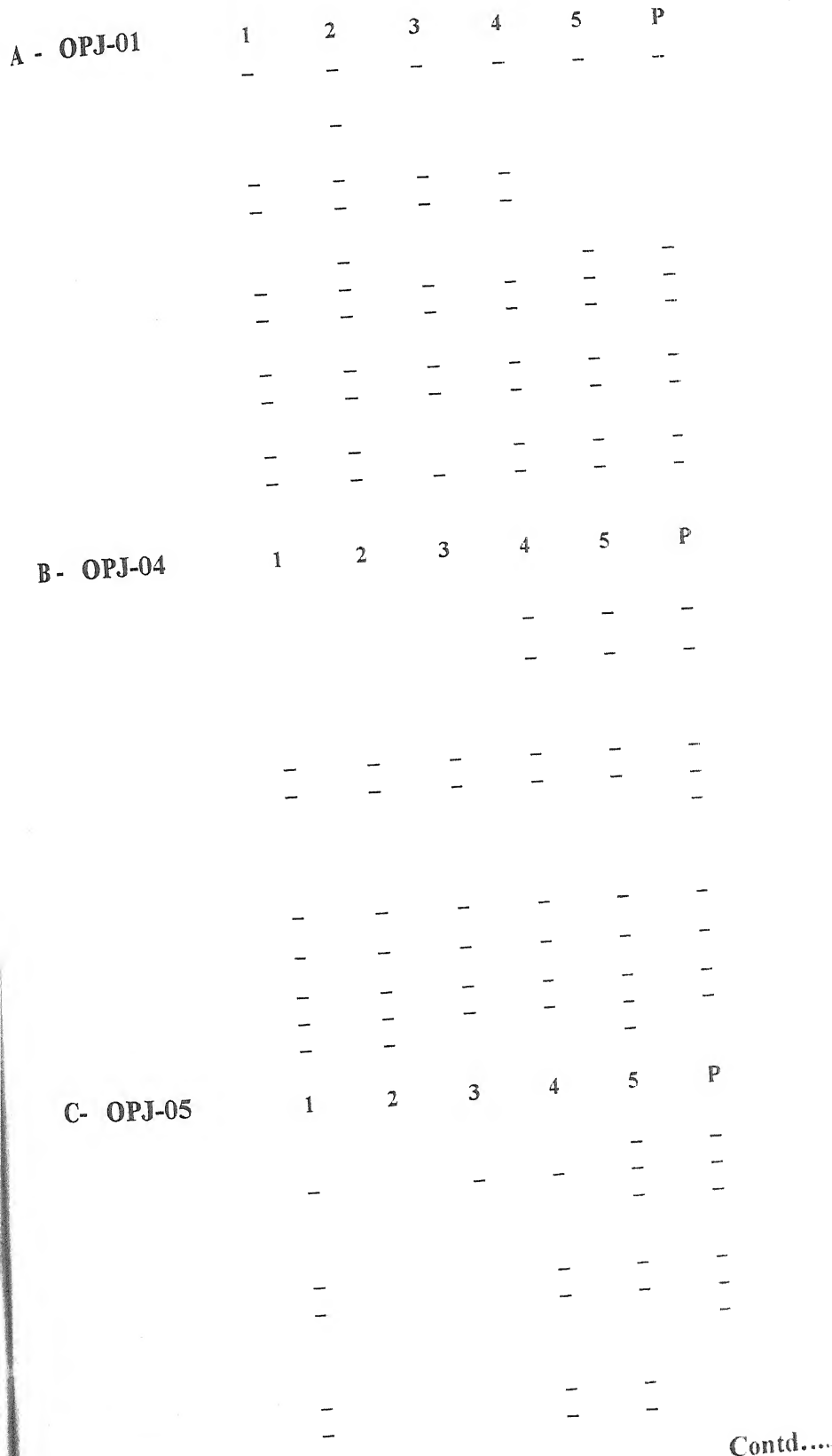


Fig.5 - OPM-04

Fig.6 - OPE-01

M - 1Kb Ladder as marker
P - Parent
S - Somaclone

Fig. - 24 : RAPD analysis of somaclones and parent



Contd.....

D - OPJ-06

1	2	3	4	5	P
–		–	–	–	–
–		–	–	–	–
				–	–
					–
					–
					–
					–
–		–	–		–
–					

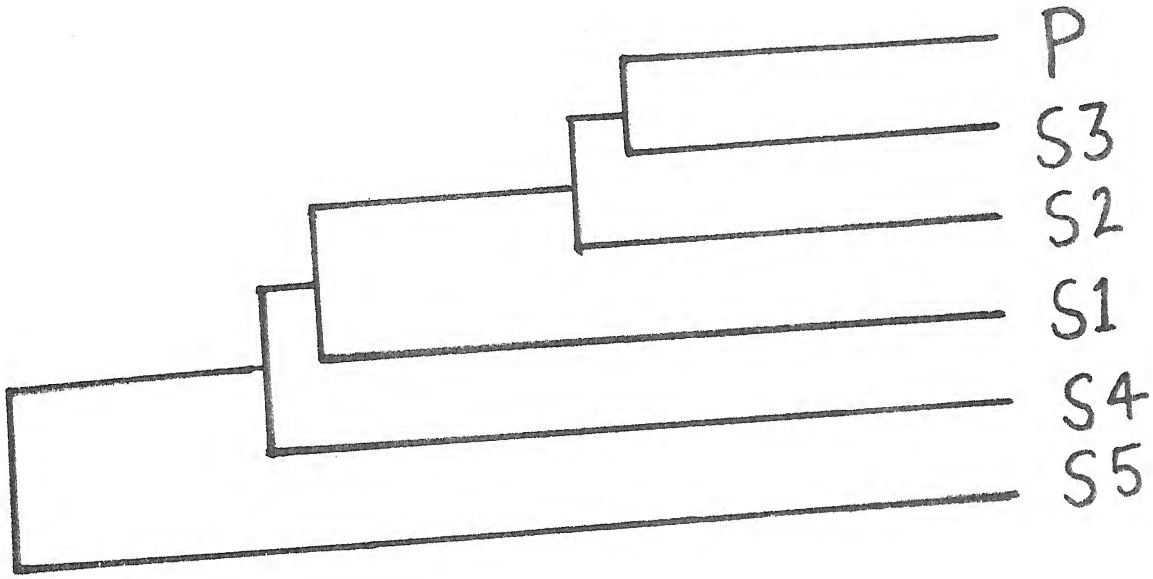
E - OPM-04

1	2	3	4	5	P
	–			–	–
–	–	–		–	–
	–			–	–
–	–	–	–	–	–
–	–	–	–	–	–
	–	–	–	–	–
–	–	–	–	–	–
–	–	–	–		

F - OPE-01

1	2	3	4	5	P
–		–	–	–	
–	–	–	–	–	
–	–	–	–	–	
–	–	–	–	–	
–	–	–	–	–	
–	–	–	–	–	
–	–	–	–	–	–
–	–	–	–	–	–
–	–	–	–	–	–
–	–	–	–	–	–

Fig. 25 – Dendrogram analysis based on RAPD banding pattern



Among all the six primers, primer OPE-01 exhibited maximum polymorphism with respect to number of alleles shared by parent with the somaclones. Above results confirmed the nature of somaclonal variation.

Dendrogram analysis based on RAPD banding pattern

Dendrogram analysis was done to understand the phylogenetic relationship among various somaclones and their parent. Genomic DNA of 5 somaclones and their parent were subjected to RAPD analysis with six primers and the results were scored to develop the dendrogram. According to the dendrograms, somaclone S3 was the closest to the parent followed by S2, S1, S4 and S5 respectively. All the 6 individuals formed a single cluster which implied closeness of somaclones to their parent. Somaclone S5 was genetically most distant compared to other somaclones (Fig.-25).

4.2.4. Forage quality traits

Nutritional composition of lucerne somaclones and their parent has been given in table-4.19. The results on proximate constituents revealed that the somaclone number 5 was found better in CP content (34.6%) than the parent (27.0%), while the parent was superior in CP content over the other five somaclones in which it ranged from 24.0 to 26.2 per cent.

Table 4.19 : Chemical analysis in somaclones and the parent

PlantNo	CP (%)	NDF (%)	NDS (%))	ADF	Cellulose	Lignin	Hemice-llulose	AI(%)
1	25.3	29.1	70.9	20.1	16.5	3.7	9.0	94.8
2	24.0	32.4	67.6	23.0	18.6	4.6	9.5	93.0
3	24.3	33.7	66.3	22.2	18.5	5.2	10.4	92.2
4	26.2	27.1	72.9	19.3	15.3	4.2	7.9	94.2
5	34.6	24.8	75.2	14.8	12.2	3.0	10.1	96.1
6	25.8	28.3	71.6	18.8	15.9	4.1	9.6	94.3
P	27.0	20.4	79.6	14.2	10.8	4.02	6.2	94.7

The cell wall contents (NDF, ADF, cellulose and hemicellulose) were relatively lower in the parent as compared to all the six somaclones. The lignin content was observed less in the somaclone number 5 (3.0%) than the parent (4.02%) which it was found more in the other four somaclones (ranging from 4.1 to 5.2%) as compared to the parent. Among all the somaclones, minimum cell wall content accumulation was recovered from somaclone number 5. Lowest level of cellulose, NDF, ADF and lignin contents were recorded in somaclone number 5 as compared to the other somaclones, the maximum NDS concentrations was observed in the parent (79.6%) followed by somaclone number 5 (75.2%), number 4 (72.9%), number 6 (71.6%), number 1(70.9%), number 2 (67.6%) and number 3 (66.3%), respectively.

However, the availability index value was maximum in somaclone number 5 (96.1%) as compared to the parent (94.7%) and the other four somaclones (94.8% to 92.2%).

Based on the quality parameters, somaclone number 5 was superior in respect of crude protein, cell wall fractions and availability index in comparison to the parent and other somaclones.

DISCUSSION

5. DISCUSSION

Medicago sativa L. belongs to family *Fabaceae* (*Leguminaceae*) and is commonly known as alfalfa or lucerne. The genus *Medicago* has a wide distribution and comprises of more than 50 species exhibiting both annual and perennial habits. Lucerne is one of the most important and the only forage crop with a very long history, known to have been cultivated before the era of recorded history. Presently, it is grown on an estimated area of more than 33 million hectares in diverse environmental conditions throughout the world (Bolton *et al.*, 1972). In India, it is cultivated in Deccan Plateau, humid belt of peninsular India and subtropical regions. In the country, it is the third most important forage crop after sorghum and berseem occupies one million hectare area of the cultivation and provides 60 to 130 tones of green fodder per hectare (Hazra, 1995). It is one of the most productive and high quality forage legume. Majority of lucerne grown worldwide is used to feed livestock and also dehydrated to produce protein supplement to be fed to the animals of all classes. It has also been recognized for its medicinal value to the sick animals. Its importance in cropping systems as a source of nitrogen and for soil improvement has been well recognized.

A number of adaptive features in lucerne, such as, its prostrate growth habit with multiple growing points close to the ground, vigorous regeneration capacity and persistence etc. have been considered quite favorable particularly for the survival under herbivore grazing conditions, as a legume.

Lucerne is a very complex species from the crop improvement point of view. It has some inherent problems for its genetic improvement, such as uncontrolled pollination, inbreeding depression, non-availability of desirable gene pool in the germplasm and lack of seed production etc. Because of these problems, the genetic improvement of this crop adopting conventional breeding methods has been quite difficult and the breeding activities have not resulted in any significant improvement in its yield potential in the last century (Rosellini and Veronesi,

2002). Hence, an increasing need for genetic manipulation of lucerne through other alternate means, namely, biotechnology has constantly been felt in the recent past.

Biotechnology has gained momentum in almost all the aspects of life sciences during the recent past. It has greatly contributed to the developments in modern genetics and led to extensive use of biological systems for advancements in the improvement of crops leading towards agricultural production throughout the world in a significant manner. Most of these advances and their use in modernizing agriculture primarily stem from their successful use for plant tissue culture systems. The basic foundation of plant tissue and cell culture systems was laid down by Haberlandt (1902) by postulating the concept of "totipotency" in plant cells. However, the establishment of the phenomenon of totipotency could be realized only after the discovery of hormonal regulation of *in vitro* differentiation and the growth in the cells of higher plants by Skoog and Miller (1957). This was further strengthened by the development of experimental procedures for successful cultures of plant tissues (Reinert, 1959), cell suspension and eventually single cell cultures (Steward *et al.*, 1958) and their differentiation leading to *in vitro* regeneration of plants.

The concept of totipotency has opened the vistas for several approaches aiming at inducing genetic modifications of plant cells and tissues, which would survive through tissue cultures and be expressed in the whole plant. Plant cell and tissue cultures have provided new options of obtaining increased genetic variability relatively rapidly. The variability generated by the use of tissue culture cycle has been referred as "Somaclonal Variation" and the somaclonal variation is now an established phenomenon (Larkin and Scowcroft, 1981). It has been established in a number of studies that genetic changes do occur in plant tissue cultures and these variations are transmitted to the regenerated plants and their progenies (Larkin and Scowcroft, 1981 and Shepard, 1981). The utilization of novel variations induced in tissue cultures has now become one of the major objectives of tissue culture based crop improvement. A series of reports on a wide range of genetic variability among plants regenerated from protoplasts, cell and callus cultures have stimulated the

interest in this type of variations for their use in genetic improvement of different crops (Shepard *et al.*, 1980; Shepard, 1981, 1982 and Thomas *et al.*, 1982). Majority of plant traits of agronomic value for which genetic variability is generated through tissue culture may provide valuable adjunct to the crop improvement. The heightened interest in somaclonal variation has emerged in part from the fact that modern genetically sophisticated cultivars could be improved by generating novel variations within co-opted gene complexes in a sequential manner by selection and for screening of plants regenerated through tissue culture and their progenies. Much of variation generated during tissue culture has been demonstrated to be heritable in many agronomically important plants together with its possible value in upgrading the genetic base in valuable varieties. The increase in genetic variability through tissue culture was first achieved in sugarcane followed by rice, wheat, lettuce and tomatoes (Larkin and Scowcroft, 1981).

Although the tissue culture of most of the species of agriculture interest has been possible, yet relatively few such species of one of the most important group, *Leguminaceae* have exhibited reproducible and efficient regeneration systems. Modern biotechnological methods, such as somaclonal variation, somatic hybridization and genetic transformation are becoming gradually very important in genetic improvement of lucerne. Optimization of tissue culture and regeneration protocols is an essential component for the successful application of tissue culture cycle in crop improvement. *M.sativa* has been found amenable for *in vitro* plant regeneration in a number of studies (Saunders and Bingham, 1972; Parrot and Bailey, 1993 and Moursy *et al.*, 1999). Nevertheless, since each lucerne plant behaves genetically different due to high heterogeneity, much genotypic variation exists for *in vitro* response in this crop. Lack of regeneration potential among the target germplasm sources might have been an early limitation to exploit somatic cell genetic improvement in lucerne. Genotypes and explants have been identified as important factors that influence *in vitro* regenerating potential of lucerne (Walker *et al.*, 1978 and Meijer and Brown, 1987). The existing technology is not yet adequate for regenerating full range of lucerne germplasm. Application of biotechnology for the improvement of popular Indian cultivars has not been attempted so far. Thus,

there is a need for identifying genotypes amenable for *in vitro* manipulation among the existing Indian cultivars or verities and optimizing their regenerating protocols so that they could be improved in their fodder yield and quality traits and also that the specific novel or alien genes could be incorporated in their genome.

In view of this background the present study was conducted in eight genotypes of *M.sativa*. The first task was to optimize *in vitro* callus production, regeneration of plants and their establishment in the field. The second task to was evaluate the *in vitro* regenerated plants (somaclones) for variations expressed in terms of important morphological or agronomic traits, isozyme patterns, RAPD polymorphism and forage quality traits.

5.1. Optimization of callus induction and morphogenetic responses of callus differentiation and plant regeneration:

A number of factors, such as, nutrient media, adjuvants and growth regulators, genotypes and explants affect callus induction and morphogenetic responses of callus for plant regeneration. Basic parameters for measurement of production of good callus are callus induction frequency and regenerability of callus. In the present study, these parameters were measured as per cent explants responding for callus induction and the quality of the callus was determined in terms of colour and texture of the calli. Callus induction and plantlet regeneration have been dependent on genotype, explant, medium and culture conditions (Vasil, 1986).

5.1.1. Callus Induction :

Excised hypocotyl, epicotyl and cotyledon form *in vitro* germinated seeds were used as explants and they were put aseptically on the culture medium. The observations on callus induction were recorded 20 days after inoculation. Differences with respect to callus induction frequency with respect to genotypes, growth regulators and media were studied.

5.1.1.1. Effect of media :

Effect of different tissue culture media on callus induction frequency was determined. The callus induction frequency in the present study revealed non significant differences among SH, MS and Blaydes media for all the three explants, namely hypocotyl, epicotyl and cotyledon.

The mean callus induction frequency was recorded maximum on MS medium followed by SH medium while Blaydes medium showed minimum callus induction response. The over all maximum callus induction responses was observed on MS medium from hypocotyls explant followed by callus induction from cotyledon explant on MS and SH medium, both. The minimum callus induction response was observed from cotyledon explant on Blaydes medium. On the basis of callus induction frequency from all the explants MS medium was found to be the best followed by SH medium.

In case of callus quality, there was no over all differences in the texture of calli raised on the three different mediums. However, the best quality of callus, in terms of its colour, was produced on MS medium followed by SH medium. The cotyledon explant exhibited the best quality of callus production followed by epicotyl.

Mariotti *et al.* (1984) found that MS medium supplemented with NAA and BA was most suitable for callus induction and growth in lucerne out of the four tissue culture media tested. Moursey *et al.* (1999) suggested that solidified MS medium with 2.0 mg/l 2,4-D and 1.5 mg/l kinetin was the best for callus induction in *M.sativa*. Arcioni *et al.* (1990) found MS medium more suitable than B5 medium in maintaining the regenerative capability of the calli in cultures of alfalfa *cv. Robot*. However, Walker *et al.* (1978) observed that callus induction and growth of the calli in lucerne was substantially improved on MS medium as compared to that on Blaydes medium. According to Kim *et al.* (1999), among SH, MS and N6 medium, The SH medium gave the highest efficiencies in callus formation and plant regeneration. Verga and Badea (1992) also found SH basal medium to be most effective among SH, MS and B5 media for callus induction in lucerne.

5.1.1.2. Effect of genotype and explant :

Callus induction response from hypocotyl, epicotyl and cotyledon explants in the eight genotypes were observed in the present study. Highly significant differences were observed among the media, genotype and their interactions with regard to callus induction frequency from hypocotyl and epicotyl explant where as in case of cotyledon explant highly significant differences were found among different media compositions and their interaction with genotypes only and no significant differences were detected with genotypes. The maximum callus induction frequency was recorded in the genotype IG-1212 followed by IL-75 from hypocotyl and cotyledon explants where as in case of epicotyl explant the maximum callus induction response was observed in the genotype LLC-9 followed by Anand-2. The minimum callus induction frequency from hypocotyl explant was recorded in the genotype LLC-3 and Anand-2 and from epicotyl explant, it was observed in the genotype A-3 and IG-1212. The cotyledon explant exhibited lowest callus induction frequency in the genotype C-10. Among the different media compositions the highest callus induction response from hypocotyl and cotyledon explants was recorded on MS medium containing 2.0 mg/l 2,4-D, 1.0 mg/l NAA and 0.2 mg/l BAP where as the maximum callus induction response from epicotyl explant was observed on SH medium supplemented with the same combination of growth regulators. SH medium supplemented with 4.0 mg/l IAA and 1.0 mg/l kinetin showed minimum callus induction response from hypocotyl and cotyledon explants where as the epicotyl explant showed minimum callus induction response on SH medium containing 1.0 mg/l NAA and 1.0 mg/l kinetin. However, no callus induction response was observed in any of the eight genotypes under study on SH medium containing 1.0 mg/l kinetin and 2.0 or 4.0 mg/l IAA from epicotyl explant only where as hypocotyl and cotyledon explants responded for callus induction in all the genotypes, though poorly.

Significant differences were observed for both the colour and texture of the callus from hypocotyl explant in different media and genotype where as in case of epicotyl and cotyledon explant the genotypic and media differences were significant with

respect to the callus colour only and for the callus texture in both these explants, the genotypic differences were found significant but the media differences were observed to be non-significant.

The best callus quality in terms of callus colour was observed from both hypocotyl and epicotyl explants in genotype IG-1212 where as the genotype AL-95-12 exhibited best callus colour quality from cotyledon explant. The best callus texture was observed in genotype Anand-2 and AL-95-12 from hypocotyl explant, in genotype C-10, IL-75 from epicotyl explant and in all the genotypes except LLC-3 in cotyledon explant. MS and SH medium supplemented with 2.0 mg/l 2,4-D, 1.0 mg/l NAA and 0.2 mg/l BAP in general showed the best callus quality from different explants.

In corroboration to the present study, the genotype specificity of the *in vitro* response of *M. sativa* has been described by several authors. (Mitten *et al.*, 1984; Brown and Atanassov, 1985 and Meijer and Brown, 1985). It is well documented that alfalfa (*M. sativa*) exhibited both intervarietal (Bingham *et al.*, 1975; Atanassov and Brown, 1984; Mitten *et al.*, 1984 and Brown and Atanassov, 1985) and intravarietal (Walker *et al.*, 1978 and Kao and Michayluk, 1981) variation in *in vitro* culture. Brown *et al.* (1984) reported wide variation among cultivars in the ability to form callus. They tested 76 lucerne cultivars for callus formation from hypocotyl and cotyledon explants. Bianchi *et al.* (1988) observed that the ability to produce callus varied widely both between and within the lines when petiole and hypocotyl explants of 20 genotypes of tetraploid and diploid populations of *M. sativa* and tetraploid populations of *M. falcata* were cultured *in vitro*. The best results from the experiment were obtained with hypocotyl and petiole explants of the tetraploid genotypes. Saunders and Bingham (1972) for the first time regenerated plants in alfalfa from the callus tissue initiated from hypocotyl, internode and ovary explants. In lucerne, various explants for callus induction and regeneration from different genotypes have been tried by different workers. Many of them have reported the superiority of petiole and hypocotyl explants over the others with respect to callus

induction (Xu *et al.*, 1990; Fujii *et al.*1992; Lai *et al.*, 1992; Yu and Pauls, 1993 and Lai and McKersie, 1994).

Mariotti *et al.* (1984) found that root and hypocotyl explants were better than those from the leaves or cotyledon for producing embryogenic callus. Nam and Heszky (1987) found that callus from hypocotyl, generally, had a higher regenerative ability than that from the other explants. Scarpa *et al.* (1991) reported that hypocotyl provided the best explants for callus production. Chen *et al.* (1987) suggested that cotyledons were a good explant source to screen for embryogenic genotypes based upon callus induction response. Hammad *et al.* (1993) found that leaves as explants always gave the best callus for subsequent regeneration. Okumura *et al.* (1993) reported that shoot tip followed by hypocotyle explant gave better callus for further regeneration.

Denchev and Atanassov (1988) found a stringent correlation between the stage of development of the initial explant and the process of dedifferentiation. They observed that interaction between explant and media or between explant and cultivars were significant. They also suggested that genotype background was most critical factor than either the medium or the nature of explant for callus induction and further regeneration. Scarpa *et al.* (1993) also found that the callus induction and regeneration responses were affected by explant source.

5.1.1.3. Effect of growth regulators :

2,4-D, NAA, IAA and cytokinins were the growth regulators for the choice of callus induction from different explant in *M.sativa.* According to Stuart and Strickland (1984), 2,4-D played important role in explant dedifferentiation and induction of callus formation. In the present study, it was observed that different concentrations of these growth regulators in different combinations influenced callus induction from hypocotyl, epicotyl and cotyledon explants differently in the eight genotypes of lucerne under study. The lower concentration of NAA with 1.0mg/l kinetin was generally found better for callus induction from hypocotyl explant in the genotypes LLC-3, AL-95-12, IL-75, Anand-2 and LLC-9. Application of 2.0 mg/l 2,4-D in

combination with lower concentrations of NAA and BAP both in MS and SH medium showed maximum callus induction response in the genotypes LLC-9, LLC-3, C-10, IG-1212, A-3, Anand-2 and AL-95-12 from hypocotyl explant. This combination of growth regulators also increased callus induction significantly from epicotyl explant in all the genotype except LLC-9. The callus induction response from epicotyl explant also improved considerably with the increase of NAA concentration in the media in the genotypes C-10, IL-75, A-3, LLC-3 and Anand-2. In the cotyledon explant, the extent of callus induction increased with the increasing concentrations of NAA in the genotype IL-75 and A-3, while it decreased in other genotypes such as C-10 and IG-1212.In general, the growth regulator combination of 2.0 mg/l 2,4-D+ 1.0 mg/l NAA + 0.2 mg/l BAP in MS or SH medium was found most suitable for callus induction frequency and the quality of callus from all the three, hypocotyl, epicotyl and cotyledon explant in all the eight genotypes used in the present study.

Kraic *et al.* (1994) also induced callus from leaf, petiole and stem segments of lucerne on a B5 based medium supplemented with 2,4-D, kinetin and NAA. Musiyaka *et al.* (1998) observed that the tissues of lucerne cultured on B5 medium with 2,4-D, kinetin and NAA initially lost its morphogenetic potential, induced dedifferentiation leading to callus formation. Wenzel and Brown (1998) obtained good callus form petiole explant of a clone of *M. sativa* var. Rangelander when exposed for 10 days to 22.6μM 2,4-D and 4.7μM kinetin on MS medium. Kim *et al.* (1999) found that in four cultivars of alfalfa, among the four media containing the same combination of growth regulators, the SH medium gave the highest efficiency in callus formation when supplemented with 3 mg/l 2,4-D, 5 mg/l NAA and 2 mg/l kinetin. According to Takamizo *et al.* (1991), BAP was more effective than any other cytokinin in terms of callus growth.

5.1.1.4. Callus growth :

In the present study, the calli were initially raised on MS medium from the three explants of the eight genotype of lucerne and luxuriantly growing calli from these explants were individually selected for callus growth rate experiment. A measured

amount of callus in each case was taken for successive subcultures at a regular interval of 20-21 days on MS medium supplemented with 2.0 mg/l 2,4-D, 1.0 mg/l NAA and 0.2 mg/l BAP and maintained upto eighty days. Callus growth rate in terms of fresh weight and dry weight of callus was measured in each case upto eighty days of calli growth. The rate of the callus growth from all the three explants from all the eight genotypes of lucerne exhibited genotype dependant growth behavior.

In case of hypocotyl explant, all the genotypes showed increase in growth of callus with the increasing duration of culture. However, in the genotype Anand-2 alone on the fresh callus weight basis and in Anand-2 and AL-95-12, on the basis of dry weight of the callus, the growth stabilized after 60 days. The maximum growth was observed after 40 days in LLC-9. Regular increase in growth rate was observed in calli of the genotypes LLC-3, C-10, IL-75, IG-1212 and A-3 even upto 80 days on both fresh and dry weight basis. The genotype C-10 had maximum dry matter content in the calli upto 40 days of subculture followed by Anand-2 and IL-75. Maximum dry matter content at 60 days of culture was recorded in IL-75 and maximum dry matter content after 80 days of culture was recorded in genotype LLC-3.

In case of epicotyl explant, all the genotypes exhibited steady and regular growth rate of callus in all the passages of subcultures. The maximum callus growth rate at 40 and 60 days in culture was exhibited by IL-75, whereas the maximum growth rate at 80 days in culture was found in genotype C-10. The maximum dry weight of callus at 40 days was observed in IL-75 and genotype AL-95-12 performed best in terms of dry callus weight at 60 and 80 days in culture. The genotype AL-95-12 had maximum dry matter content accumulated at 40 days of culture while genotype Anand-2 and AL-95-12 showed maximum dry matter content in their calli at 60 days of culture. The genotype AL-95-12 also showed maximum dry matter content in the calli upto 80 days of culture.

In case of cotyledon explants also, callus growth rate in all the eight genotypes increased with increase in days in subcultures on fresh callus weight basis but on the basis of dry callus weight, the callus growth rate increased upto 60 days only. The genotype IL-75 and LLC-3 had maximum dry matter content upto 80 days of culture and the genotype IG-1212 had maximum per cent dry matter content at 60 days and the 80 days of culture.

The results of callus growth rate in terms of fresh and dry callus weights and the per cent dry matter content in the calli in all the eight genotypes during various passages of subculture indicated that there was a marked variation in the response of these parameters as effected by different genotypes. In general, al the genotypes showed steady growth rate upto 40-60 days. There was an initial increase in the per cent dry matter content upto 40 days of culture followed by its gradual decrease till 80 days of callus growth. The optimum callus growth, for all the three parameters studied, was observed between 20 and 40 days of callus cultures.

The variability for *in vitro* callus proliferation in terms of callus fresh weight, callus growth rate and callus production in lucerne was also found by Brown and Atanassov (1985) in corroboration to the present study. They evaluated numerous lucerne cultivars to determine the role of genetic background in the *in vitro* cultures of *Medicago*. A wide range of callus yield was observed among cultivars ranging from 62 mg for *cv*. Chilan hypocotyl explant to 720 mg for *cv. Angus* cotyledon. Wide variation for callus growth among genotypes, within some cultivars was also detected. Mroginski and Kartha (1984) also observed that callus initiation and *in vitro* callus proliferation were dependant upon several factors including genotype, explant tissue, culture medium, the environment under which the explant tissue was grown and other *in vitro* culture conditions. They observed that genetic variability existed for both callus initiation and *in vitro* callus proliferation within plants species including *M.sativa*. In an evaluation of several *M.sativa* cultivars by Matheson *et al.* (1990) indicated that all the plants within these populations were not capable of callus formation. The importance of the genetic variance for determining callus production has also been noted in other species such as *Zea mays* (Beckert and Qing,

1984) and *Trifolium pratense* (Keyes *et al*, 1980). Both Beckert and Qing (1984) and Keyes *et al* (1980) found significant reciprocal effects for callus growth.Kielly and Bowley (1997) observed that it was possible to develop high *in vitro* callus producing populations in alfalfa germplasm.

5.1.2. Morphogenetic responses of callus differentiation and plant regeneration :

Morphogenetic differentiation towards somatic embryogenesis in *M.sativa* was first reported by Saunders and Bingham (1972) in one plant of cultivar Saranac. Since then, regeneration of somatic embryos in alfalfa has been achieved from cells in suspension cultures (McCoy and Bingham, 1977), long term callus and suspension cultures (Stavarek *et al*., 1980) and protoplasts (Dos Santos *et al*., 1980; Kao and Michayluk, 1980; Johnson *et al*., 1981 and Mezentsev, 1981). In the present study, based on callus induction response, callus quality and callus growth rate, the calli from hypocotyl, epicotyl and cotyledon explants of all the eight genotypes of lucerne under study were cultured on regeneration media for inducing *in vitro* regeneration. The experiments for *in vitro* regeneration were conducted following two pathways, namely, 1) Morphogenetic response of calli directly on the regeneration media and 2) Morphogenetic response of calli on the regeneration media following auxin shock treatment.

5.1.2.1. Morphogenetic response of calli directly on the regeneration media :

MS and SH basal media with different concentrations of auxins and cytokinins were used for studying morphogenetic response of the calli. In total, 48 each of different MS and SH based regeneration media supplemented with kinetin (0.0 mg/l – 8.0 mg/l), BAP (0.0 mg/l – 4.0 mg/l), NAA (0.0 mg/l – 1.0 mg/l) and IAA (0.0 mg/l – 1.0 mg/l), in various combinations were used. The selected calli from various explants of all the genotypes under study were transferred to various regeneration media for inducing morphogenesis towards regeneration.

Different types of morphogenetic responses of callus differentiation and the quality of calli developed on various 96 combinations of MS and SH based regeneration media were observed. Most of the media combinations yielded only different types of callus quality ranging from white, light yellow, yellow, yellowish green, yellowish white and green to little bit brown callus in colour and nodular to friable in texture.

Morphogenetically differentiating types of calli of different colours ranging from white, light yellow, yellowish green, greenish white and green were developed on certain MS and SH based regeneration media combinations containing 0.5 mg/l - 1.0 mg/l kinetin alone or 0.5 mg/l kinetin with 0.5 mg/l BAP together with 0.5 mg/l - 1.0 mg/l NAA or 0.5 mg/l - 1.0 mg/l IAA. MS and SH based regeneration media containing higher concentrations of kinetin ranging from 4.0 mg/l - 8.0 mg/l with or without addition of 1.0 mg/l – 2.0 mg/l BAP together with 0.5 mg/l – 1.0 mg/l NAA or IAA also led to the formation of morphogenetically differentiating types of calli from all the explants in most of the genotypes. However, when MS and SH medium without containing any growth regulators were used as regenerating media, the calli of all the three explants of all the eight genotypes under study turned brown within 20 days of their transfer to these regeneration media without showing any morphogenetic differentiation of any type except in case of the callus of the cotyledon explant of LLC-3 genotype only. This callus from the cotyledon explant of LLC-3 genotype when placed on MS basal regenerating medium, developed somatic embryos within 3-4 weeks after inoculation. These somatic embryos developed through various stages of somatic embryogenesis, however, their subsequent the growth and regeneration into plantlets were arrested.

5.1.2.2. Morphogentic resonse of calli on regeneration media following auxin shock treatment :

The selected calli from different explants of various genotype under study were transferred on auxin shock treatment media for four days following the method of Romagnoli *et al.* (1996) and then transferred and grown subsequently on four

different regeneration media sequences, namely, SHKI, MSKI, SHPKI and MSPKI, separately for regeneration. The calli grown on SHKI and MSKI regeneration media sequences, developed into various types of quality of calli ranging from yellowish white, yellowish green, light green to yellowish brown and did not show any kind of regeneration response. The effect of basal media was evident on these calli. Greening of calli was observed in MSKI whereas the calli turned yellowish white and subsequently became brown and non regenerative on SHKI media sequence. However, after auxin shock treatment when calli were transferred to proline supplemented MS or SH media (MSPKI, SHPKI), globular shaped somatic embryos were differentiated within 6-7 days of culture and the cultures were full of these globular structures within 18-20 days. These globular shaped somatic embryos also remained arrested at the stage only but when these calli were transferred to kinetin and IAA sequense of MSPKI and SHPKI media separately, the greening of these callus was observed on MS based medium while the callus remained yellowish and became brown on SH based regeneration medium. The green calli of all the genotypes subsequently developed shoot primordia on 4.0 mg/l kinetin and 1.0 mg/l IAA step of MSPKI regeneration sequence. On further increasing the kinetin to 8.0 mg/l in the next step of MSPKI regeneration sequence, shoot bud organogenesis was observed in the calli of hupocotyl explant of the genotypes IL-75, C-10, IG-1212 and AL-95-12, cotyledon explant of A-3 and LLC-3 and epicotyl explatnt of IL-75 only. At this higher level of kinetin in MSPKI regeneration sequence, the shoots started turning yellowish brown instead of further development of shoots, but, when these developing shoots were transferred to reduced kinetin concentration (2.0 mg/l), healthy green shoot development and shoot bud multiplication was observed. The shoot bud development was improved on their transfer to MS basal medium of MSPKI media sequence. The proline supplementation and the effect of basal medium were found critical as regeneration occurred on proline supplemented MSPKI regeneration sequence only and no regeneration was observed on MSKI or SHKI sequences which were devoid to proline, or even on proline supplemented but SH based SHPKI regeneration media sequence. The regeneration on MSPKI media sequence occurred mostly through shoot bud organogenesis and varied among different explants and genotypes. The regeneration of shoots per calli was maximum

form the calli of cotyledon explant of LLC-3 genotype followed by hypocotyl explant of IL-75. The minimum efficiency of regeneration was observed from the calli of epicotyl and cotyledon explants of IL-75 and A-3 genotypes respectively. The calli from hypocotyl explant showed maximum response for regeneration followed by that of cotyledon and least response for regeneration was exhibited from the calli of epicotyl explant.

Several authors (Meijer and Brown, 1985 and Chen and Marowitch, 1987) have also reported that only a few genotypes in certain cultivars have been found to possess the regeneration capacity. The plant regeneration studies from *in vitro* cultures have shown that not all lucerne germplasm were able to produce somatic embryos and plantlets, the frequency of induction of somatic embryogenesis also varied among various cultivars (Bingham *et al.*, 1975 and Brown and Atanassov, 1985). This variation was observed even among the genotypes of a single cultivar (Kao and Michayluk, 1981; Phillips, 1983 and Mitten *et al.*, 1984). Even though the frequency of regenerating genotypes within a cultivar was high, much variation existed in the efficiency of regeneration among them (Mitten *et al.*, 1984). This was attributed to the intervarietal and intravarietal heterogeneity in alfalfa. Fuentes *et al.* (1993) evaluated *in vitro* embryogenic response in 9 lucerne varieties and found that all the varieties, except San Joaquin II, gave a positive response in one or more of the protocols tested. Arcioni, *et al.* (1989) suggested that it was necessary to identify and isolate regenerating genotypes before using *M. sativa* in plant genetic manipulation studies owing to the higher degree of genotypic variation for regeneration. The range of germplasm from which plant regeneration has been reported is, however, vary narrow. The numbers of regenerative genotypes from which plant populations have been established and evaluated are even more limited. The clones from cultivar Regen S developed by Bingham *et al.* (1975) have been used almost exclusively for regeneration studies (Walker *et al.*, 1978, 1979; Hartman *et al.*, 1984 and Stuart and Strickland, 1984). The regeneration frequency was affected by the interactions, medium x genotype (Brown and Atanassov, 1985) and explant x genotype (Novak and Konecna, 1982). Brown *et al.* (1984) reported wide variation among cultivars in the ability to form somatic embryos. Somatic

embryo formation occurred only in 34 per cent of cultivars, when 5 medium protocols using 4 cultivars were compared by them. However, Chen *et al.* (1987) observed that some genotypes produced somatic embryos regardless of medium protocol or explants source. Suginobu *et al.* (1991) concluded that genotype background was a more critical factor for somatic embryogenesis than either the medium or the nature of the explant. Varga and Badea (1992) suggested that the application of tissue culture techniques for alfalfa improvements required screening within specific cultivars in order to identify genotypes capable of regenerating entire plants.

In lucerne, various explants for callus induction and regeneration from different genotypes have been tried by different workers. Many of them have reported the superiority of petiole and hypocotyl explants with respect to regenerability (Piccioni *et al.*, 1996). Lupotto (1983) induced cyclic production of embryoids through secondary embryogenesis from cells derived from the hypocotyl callus. The high probability of predicting of embryogenic genotypes based upon cotyledon callus suggested that cotyledons were a good explant source to screen for embryogenic genotypes (Chen *et al.*, 1987). Okumura *et al.* (1993) reported that shoot tip explant had better ability to undergo somatic embryogenesis. However, the competence of the different tissues in the regenerative pathway has not been completely understood (Finstad *et al.*, 1993). Scarpa *et al.* (1993) found that hypocotyl derived callus were the best regenerating tissue in *M. polymorpha.* The concentration and combination of growth regulators have been known to govern plant regeneration. For most the legumes, the early stages of somatic embryogenesis have been induced by exposure of tissues to 2,4-D with or without association of other growth regulators (Ammirato, 1983), which was in corrboration with present study. In many legumes, as was observed in the present study also, the removal of 2,4-D and exposure to media lacking hormones or with various combinations of cytokinin and other auxins has led to maturation of embryoids and shoot formation. The inductive role of 2,4-D in regeneration of organogenic shoot buds and somatic embryo formation has been demonstrated by several authors (Saunders and Bingham, 1975; Walker, *et al.*, 1979 and Walker and Sato, 1981).

The induction periods varied with the concentration of 2,4-D and plant species which ranged from 6 months Mitchell cultivar of soybean (Christianson *et al.*, 1983) to as little as 3 to 4 days for alfalfa (Walker and Sato, 1981 and Brown and Atanassov, 1985). Finstad, *et al.* (1993) also demonstrated the requirement for the acquisition of competence prior to the induction of embryogenic pathway. In a study by Shetty and McKersie (1993), somatic embryogenesis was induced in petiole derived culture in the presence of 1mg 2,4-D and 0.2 mg/l kinetin and embryo elongation occurred when embryogenic calli were transferred to hormone free medium. Lupotto (1983) showed that although the initiation of somatic embryogenesis started on simple hormone-free medium, a more complex medium with yeast-extract was needed to propagate embryogenic cultures. However, the induction phenomenon was not found specifically to 2,4-D only (Kao and Michayluk, 1981) as NAA has also been observed replacing 2,4-D in several cases. Won *et al.* (1999) found that somatic embryos were formed from callus obtained from hypocotyl explants of *M. sativa cv.* Vernal on MS medium containing 4 mg/l 2,4-D and 0.1or 0.5 mg/l kinetin. Nolan *et al.* (1989) reported that induction of embryo formation occurred on a medium containing 10μM NAA and 10μM BAP and embryo maturation was promoted after transfer to a medium containing 1μM NAA and 10 μM BAP and shoot development or occasional plantlet development occurred on a subsequent transfer to 0.1 μM NAA and 1μ M BAP. Zhang *et al.* (1995) cultured callus of *M.sativa cv.* Jining in liquid MS medium containing 4 mg 2,4-D and 1 mg BA/litre. Leaves and buds were induced after subculturing. Takamizo *et al.* (1991) concluded that the UM medium containing 4mg/l 2,4-D supplemented with BAP rather than kinetin was more effective for somatic embryogenesis in hypocotyl derived calli of *M.sativa cv.* Tachiwakaba. Parrott and Bailey (1993) produced somatic embryos in five genotypes out of the callus cultures of 300 genotypes of *M.sativa* on Blaydes medium containing 10.74 μM NAA, 11.42 μM IAA and 9.29 μM kinetin from leaf, petiole and internodes explants. Shao *et al.* (2000) developed two protocols for the regeneration of tetraploid lines of alfalfa. In the first regeneration system, leaf explants were

incubated on MS medium supplemented with 2,4-D and kinetin for callus formation and subcultured onto growth regulator-free MS medium to induce direct somatic embryogenesis. In the second regeneration system, the inoculated explants were incubated on B5 medium with hormones to produce somatic embryos *via* embryogenic callus. Saunders and Bingham (1972) regenerated plants in alfalfa from callus tissue initiating from hypocoty, internode and immature ovary through both organogenesis and embryogenesis following a two step method. The calli were developed on B5 basal medium in lucerne, morphogenesis was induced on the same medium containing benzyl adenine phosphate with subsequent placement on a hydrate with growth regulators which ultimately led to formation of shoots (Kraic *et al.*1994). Saunders and Bingham (1972) observed that the callus on its transfer to a medium containing inositol and yeast extract differentiated into shoot buds profusely and most of this grew into plants. According to Romagnoli *et al.* (1996) observed that the best media for somatic embryo production was MS medium + 10 µM 2,4-D + 4.6 µM kinetin. After the auxin shock, when the calli were transferred to MS medium containing 10-20 mM NH_4^+ and 30 mM proline, a large number of somatic embryos were produced which were grown to plants on MS or half strength of MS medium.Addition of 10-25 mM proline and 0.1-0.5 mM thioproline in the medium stimulated 2,4-D induced somatic embryogenesis. Stuart and Strickland (1984 a, b) also found that the addition of L-proline to SH medium, increased embryoid formation. Further development of embryoids occurred in media supplemented with either yeast extract or in MS based media containing high levels of nitrogen. Present study has been in confirmation on with that of Stuart and Strickland (1984) who also observed that the proline-enhanced regeneration of somatic embryos. The Optimum regeneration occurred when 10mM proline and 25mM ammonium was added to the regeneration medium. Walker and Sato (1981) suggested that exogenously supplied ammonium ions were critical to *in vitro* morphogenesis. Shetty and McKersie (1993) suggested that addition of proline, thioproline and potassium enhanced the size of embryogenic callus significantly subsequent embryo formation along with higher number of cotyledonary embryos.

In corroboration to the present investigation, Walker *et al.* (1978) also obtained regeneration through shoot bud organogenesis in *M. sativa* L. *cv.* 'Regen' by transferring callus from induction medium containing growth regulators to a regeneration medium lacking of growth regulators. They suggested that the determination of organ type occurred principally on regeneration medium. During organogenesis in alfalfa, the process of organ induction might initiate on induction medium and the determination of organ type had taken place on regeneration medium. Churova (1981) observed that growth centers were induced and stalk meristems differentiated and developed into rootless plants (shoot stocks) in MS medium as in case of present study also. However he also successfully attempted to induce shoot bud organogenesis in callus cultures of *M. sativa* on Blaydes medium. Musiyaka *et al.* (1998) cultured callus of *M.sativa* on B5 medium with 0.5mg/l 2,4-D and 50mg NAA/l and restored morphogenetic potential. Buds and roots formed after 30 days of culture on a medium inducing morphogenesis. Nikolic *et al.* (1985) observed that callus induction, bud formation, shoot elongation and rooting in lucerne were obtained on sequence of media with different hormone additions.

Bingham *et al.* (1975) observed that embryogenic capacity was controlled by two genetic loci and it was possible to incorporate it into cultivars by means of recurrent selection. Increase regeneration was found in the Regen S cultivar of alfalfa, developed by two cycles of recurrent selection for regeneration capacity. Reisch and Bingham (1980) found that in diploid alfalfa, bud differentiation from callus by controlled by two dominant gene and both were required to present in order to obtained more than 75 % regeneration. Wan *et al.* (1988) studied 7 tetraploid lucerne cultivars and they also suggested that regenerability in this system was controlled by 2 complementary dominant genes, both were necessary for regeneration. Gene dosage influenced regeneration efficiency and cytoplasmic effects influenced the interaction between callus induction medium and regenerability. Walton and Brown (1988) found evidence of cytoplasmic inheritance for extent of embryogenesis in 2 reciprocal crosses of *M.sativa*.

5.1.2.3.Histology of morphogenetic responses

In the present study, morphogenetic potential towards regenerability of different types of calli was observed and it was found that only whitish or greenish white and granular or nodular types of calli were capable of regeneration. The cell masses among the calli potentially capable of morphogenetic differentiation were composed of compact and round shaped cells of comparatively small and uniform size with densely staining cytoplasm. These regenerating cells were without intracellular spaces and exhibited meristematic activity. These types of cells were also noticed in regenerating calli by Kohlenbsch (1978) and Vasil and Vasil (1982). Vasil and Vasil (1981), observed that embryogenic calli were characteristically compact, much organized and white to pale yellow in colour. These regenerating calli when induced for morphogenetic differentiation on various regeneration media exhibited both pathways of regeneration, that is, *via* somatic embryogenesis and shoot bud organogenesis. The most frequently noted morphogenetic response in the present study was the organogenic induction of shoot buds while somatic embryogenesis was quite ephemeral. The histological examination for occurrence of somatic embryogenesis and shoot bud organogenesis in the present study revealed that the developing somatic embryos exhibited bipolar meristematic structures with a plumule region at the distal end and radical region at the proximal end of these structures. Such somatic embryos were largely globular, heart shaped or torpedo shaped, having cotyledonary protuberances. The most commonly occurring and stable morphogenetic response towards the regeneration from the regenerating calli was through somatic bud organogenesis. The histological by such unipolar meristematic outgrowths appeared on the surface of these calli during organogenesis which soon assumed the structure of shoot buds. The regenerating shoot buds were typically by similar to the vegetative shoot apex in their morphology and histology examination having clearly discernible tunica-corpus organization and bearing laterally subtending leaf primordia. Such shoot buds developed in to vegetative shoots bearing leaves laterally and were like shoot stumps, which had vary little or no shoot primordia. Similarly, morphogenetic structures developing in the regenerating calli were also observed by Bond and

Webb (1989) in their study with *Trifolium repense*. Ammirato (1983), also found the formation of bipolar structures, resembling zygotic embryos, with both a shoot and root meristem during their study of somatic embryogenesis in lucerne as opposed to organogenesis in which only shoot and root formation was induced. Initially, somatic embryos were thought to arise exclusively from single cells (Haccius, 1978). Histological examination of embryogenic tissues showed that the embryos arose from single cells of the parental explant (dos Santos *et al.*, 1983). Dos Santos *et al.* (1983) also suggested that embryoids were organized from groups of apparently homogenous meristmatic cells on organised callus, white or friable callus and were originated from single embryogenic cells distinguishable from the nonembryogenic cells by staining reactions. They provided evidence for the origin of embryoids from single cells in the epidermis of cotyledons, hypocotyls and roots of induced plantlets as well as in friable callus of *M. sativa*. Iantcheva *et al.* (1999) developed somatic embryos from *M. truncatula, M. littoralis, M. murex and M. polymorpha* and confirmed the nature of the directly formed somatic embryos by histological analysis.

5.1.2.4. Rhizogenesis:

The shoots regenerated on MSPKI regeneration medium sequence from the calli of various explants in six genotypes, LLC-3, IL-75, A-3, C-10, IG-1212 and AL-95-12 lacked roots, possessing only shoot stumps at their basal ends which were surrounded by the callus from which they arose. These shoot stumps along with a little bit a surrounding callus on their transfer to the same hormone free MS medium showed very poor development of roots which was limited to LLC-3 and IL-75 genotypes only. Hence, they were transferred on eight different rooting media, 4 each based on MS and SH basal salts supplemented with 0.5 mg/l kinetin, 2.0 mg/l NAA and with or without 2.0 mg/l IAA and with or without 3 g/l activated charcoal. The best response for root induction and development was observed in the shots of LLC-3, IL-75, IG-1212 and C-10 genotypes on the SH based root induction medium containing 0.5 mg/l kinetin, 2.0 mg/l NAA, 5.0 mg/l IAA and 3g/l activated charcoal. The developments of roots were comparatively vigorous in LLC-3 and IL-75 than IG-1212 and C-10 genotypes and no rooting response was

observed in the genotype A-3 and AL-95-12 in any of the eight rooting media. Kraic *et al.* (1994) could induce the roots on the *in vitro* developed shoots on rooting medium in lucerne. Liu *et al.* (1993) could produce adventitious roots along the buds on the differentiation media from the calli derived from hypocotyl and cotyledon of *M.sativa*. Scarpa *et al.* (1993) observed rooting and plantlet development on a medium containing 2-ip and IAA during regeneration of plants from hypocotyl derived calus in *M.polymorpha*. However, Iantcheva *et al.* (1999) observed in *M.truncatula, M. littoralis, M. murex and M. polymorpha*, that the shoots regenerated *in vitro* easily develop root system on a medium with reduced level of macro elements and sucrose.

5.1.2.5.Survival of plantlets :

Habituation of the *in vitro* grown plantlets to the external harsh conditions as been observed as a gradual process. In the present study, the regenerated plantlets with well developed shoots and roots were taken out of the culture flasks and after washing their roots thoroughly with sterile water they were transferred in small plastic pots filled with autoclaved soilrite and well soaked with ¼ strength MS basal salt solution containing no growth regulators and sucrose. Sufficient moisture regime and controlled temperature and photoperiodic conditions were maintained around the pots for few days. The surviving plants were then transferred to larger pots containing soil, sand and FYM (1:1:1 v/v) and kept in diffused light at room temperature for 8-10 day before their transfer to field. The plants with well developed roots and shoot were obtained in LLC-3, IL-75, C-10 and IG-1212 genotypes only. In genotypes C-10 and IG-1212, none of the *in vitro* regenerated plants could with stand even the first step of hardening. However, in case IL-75, out of 30 regenerated plantlets, only 12 could survive in the first step of hardening and none of could survive in the subsequent stage of hardening and acclimatization. Whereas, Out of 60 regenerated plants of LLC-3, 52 survived in the first step of hardening and only 42 plants could develop to maturity in the field condition. All these 42 *in vitro* regenerated plants belonged to LLC-3 genotype regenerated from cotyledon explant. Nagarajan *et al.* (1986) also established regenerated lucerne plants in soil. Rooted plantlets were also transferred in to soil and adapted to field

condition gradually by Kraic and Hzik (1994). However, regenerated plantlets of lucerne were transplanted into soil acclimatized in a grown chamber for two weeks until the plants reached 20 cm and then moved to the green house (Moursy *et al.*, 1999), whereas embryo were planted directly into soil plotting mix under non sterile condition without exogenous nutrients (Fujii *et al.*, 1999).

Plantlets were transferred to soil and maintained for 3 weeks in a grown cabinet (216μ mol m^{-2} s^{-1}, 23 ± 1^0C, 12-h photoperiod, 80 % R.H) by Scarpa *et al.* (1993). However, Nolan *et al.* (1989) transferred the plantlets to sterile sand supplemented Hoaglands nutrients solution for further growth, where as according to Atanassov and Brown (1984), mist chamber was necessary for plants and after 7-12 days in the mist chamber plants could successfully be potted and moved to the green house. According to Saunders and Bingham (1972), plantlets were potted in sterilized soil inoculated with rhizobium and grown briefly in the green house before transfer to the field.

5.2. Somaclonal variation :

Somaclonal variation commonly appear in *in vitro* plants regenerated *via* callus phase. One of the applications of tissue culture is the exploitation of genetic changes occurring in plants regenerated from callus and protoplasts for producing agronomically useful variants (Larkin and Scowcroft, 1981 and Evans and Sharp, 1983). Plants regenerated from tissue culture might quite often vary from the tissue donor plant in one or more characteristics. This variation was termed as "somaclonal variation" by Larkin and Scowcroft (1981). There has an emense utility of somaclonal variation for inducing genetic variability in lucerne since a large amount of natural variability is known to be present in alfalfa, but it has been very difficult to improve forage yield potential in comparison to cereals and food crops (Bingham, 1981). Because of these problems, the genetic improvement of this crop adopting conventional breeding methods has been quite difficult and the breeding activities have not resulted in any significant improvement in its yield potential in the last century (Rosellini and Veronesi, 2002). Induction and the possible utilization of

somaclonal variation in the genetic improvement of alfalfa could be derived from comparison of ranges of variations observed in the somaclones and the original populations.

5.2.1. Morphological variations in somaclones :

Among all the somaclones, the plants developed from the callus of cotyledon explant of the genotype LLC-3 only could survive in the field and develop up to maturity. The performance of these forty-two cotyledon derived somaclones of LLC-3 was studied for various morphological traits of agronomic importance as compared to the mean values for the respective trait of the parent and number of superior and inferior somaclones for the respective trait, frequency of somaclonal variation and the frequency distribution of the somaclones for various traits were determined. The regenerated plants, the somaclones, exhibited bi-directional variation for most of the morphological traits. The mean performance of all somaclones deviated considerably for most of the traits as compared to the parent. Overall performance of the somaclones was found better over to the parent with respect to the number of nodes and internodes, stem girth and flower size. The maximum number of superior somaclones was recorded for stem girth followed by for number of nodes and length of internodes. The minimum number of superior somaclones over to parent was observed for per cent dry matter content followed by fresh leaf weight and flower length. The range of bi-directional variation was substantial in case of plant height, branches per plant, fresh and dry weights of leaf and stem, total green weight, total dry weight, dry matter content and flower size. Frequency of variation in the somaclones varied considerably for various morphological characters while for stem girth and dry weight of stem most of the somaclones deviated significantly from the parent and the minimum variation was noticed with regard to per cent dry matter content. The frequency distribution of somaclones for various morphological traits as per the variation occurred in both the directions in terms of the mean values of the parent and the similarity was exhibited in the somaclones with respect to parent for most of the characters under study.

Somaclonal variants for qualitative genetic alternations (Bingham and McCoy, 1986), disease resistance (Latunde-Dada and Lucas, 1983 and Johnson *et al.*, 1984) and quantitative traits such as herbage yield (Johnson *et al.*, 1984 and Pfeiffer and Bingham, 1984) have already been reported in alfalfa. Nagrajan and Walton (1989) found that plant height, stem girth, number of nodes, per stem and internodes length were lower in one somaclone than the original populations. Somaclonal variation is a common phenomenon and an array of variation involving flower colour, leaf morphology, branching patterns, fertility, regeneration ability, vigor and ploidy level has been observed among regenerated plants (Reisch and Bingham, 1981; Bingham and McCoy, 1986 and Groose and Bingham, 1986). As in case of present study variation in morphological and biochemical traits were also studied in regenerated lucerne plants (Deineko *et al.*, 19997). Arcioni *et al.* (1988) found that somaclonal variation was genotype specific.

In a field study of herbage yield of 32 diploid and 16 tetraploid regenerates of HG2 alfalfa revealed variants for herbage yield (Reisch and Bingham, 1981). These results were in corroboration to the present study. Herbage yield as considered as quantitative genetic trait as controlled by many genes. Somaclonal variants of alfalfa were also noted among the alfalfa plants regenerated from the callus (Saunders and Bingham, 1972). Johnson, *et al.* (1980) reported that thirty-two regenerated plantlets were phenotypically different from the plant of the lucrene variety Regen S1 from which they were regenerated. Groose and Bingham (1984) obtained plants from hypocotyl derived callus of two tetraploid alfalfa genotypes bred to be heterozygous for four heritable traits and 21 per cent of the regenerants were variants for one or more characters. Hartman, *et al.* (1984) found that somaclonal variation for disease resistance in alfalfa was genetically transmissible and appeared to be due to dominant mutation. Ptackova *et al.* (1988) observed significant differences in water holding capacity in 40 somaclones from a single *M.sativa* plant. Some of them combined drought resistance with high chlorophyll content. Safarnejad *et al.* (1996) observed that one somaclone when compared with the parent line of lucerne showed increased salt tolerance, greater accumulation of proline and a greater increase of antioxidant enzyme, glutathione reductase.

However, Nikolic *et al.* (1986) observed that the regenerated plants did not differ from normal plants of the variety in their morphological traits and yield.

Mechanisms of somaclonal variation identified in alfalfa include changes in chromosome number and structure, dominant and recessive mutations, transposable elements and changes in chloroplasts and mitochondria. A variant may carry more than one nuclear or cytoplasmic mutation.

5.2.2. Biochemical characterization of somaclones :

Isozymes of PGI, PGM, G6PDH, AAT, SOD and esterase were analysed for 6 somaclones with their parent using PAGE technique. Somaclones differed among themselves with their parent for isozymes of PGI, PGM, G6PDH, AAT SOD and esterase. High level of genetic variability within and between populations of *M.intertexta* than *M.ciliaris* was reported by Cherifi, 1996. In another study on protoplast fussion, the hybrid nature of the selected calli was confirmed by isozyme analysis (Pupilli *et al.*, 1991). Similarly, Pupilli *et al.* (1992) confirmed hybrid nature of regenerants by isozyme analysis.

This is the first report on somaclonal variants substantiated by isozyme polymorphism in lucerne. Where as several reports have established somaclonal variation on isozyme variation (Mangolin *et al.*, 1997; Binsfold *et al.*, 1996 and Pramanik *et al.*, 1996). However, Richard *et al.* (1995) reported that results from isozyme system could not differentiate significantly the hybrid larch plants regenerated from tissue culture from their stocklings and the ortet. Pramanik *et al.* (1996) found a new band of superoxide dismutase in multiplying shootlets which was, according to them, a *denovo* variation or due to post transcriptional modification of the gene. Similarly Mangolin *et al.* (1994) found that IDH, ACP, PER and EST enzyme systems were good markers for investigating possible genetic variations. However, the present study indicated PGI, PGM, G6PDH, AAT, SOD and EST isozymes as good enzyme systems for detecting somaclonal variation.

Many studies have reported isozymic differences between somaclones developed using various explants. Khavkin (1991) have found that some loci more susceptible to somaclonal variation than others. His data indicated a specific control system whereby ordering tissue and organ specific isozyme spectra might be affected by somaclonal variation at the early stages of a regulatory cascade. Martelli *et al.* (1993) could distinguish rootstocks and regenerants in apple based on isozyme banding patterns.

Dendrogram analysis :

Dendrogram analysis from the data analysed from six isozymes indicated 3 groups of six somaclones and parent based on similarity index. Our study indicated S1 and S2 as closer to parent than others and S5 was the most distant somaclone. This analysis further substantiated somaclonal variation as somaclones clustered into 3 groups. Cherifi (1996) also reported 2 taxa based on cluster analysis between populations of *M.intertexta* and *M.ciliaris*. Whereas, Zhang and Hao (1993) grouped lucerne genotypes grown under different environmental conditions into 3 groups based on banding patterns of peroxidase and esterase isozymes. However, the present study is the first report of such an analysis among somaclones of *M.sativa*. Utility of results of such dendrogram analysis could be to use distant somaclones as targets for cross hybridization.

5.2.3. RAPD analysis of somaclones :

To further substantiate the results obtained from isozyme analysis, DNA markers such as random amplified polymorphic DNA were analysed among somaclones of lucerne. Results indicated polymorphism for all the primers used further confirming somaclonal variation. RAPD markers are more efficient and reliable to characterize variation as they indicate genomic differences without getting affected by the environment and post transcriptional modifications Similar opinion was reported by Echt *et al.* (1992) who found RAPD markers useful for the rapid development of genetic information in species like lucerne where little information currently exists or it is difficult to obtain. Similarly, Phan *et al.*, 1996 also found extensive DNA

changes in cultured cells demonstrating occurrence of somaclonal variation in the material used to produce protoplasts for the gene transfer.

In the present study, the maximum polymorphism was recorded from OPE-01 primer with respect to number of alleles showed by parent with the somaclones. Yu and Pauls (1993) also identified RAPD markers linked to gene controlling somatic embryogenesis in *M.sativa*. The segregation data indicated that the marker was linked to A locus. However, in this study, segregation analysis of somaclones using RAPD in still to be studied. Another study by Piccioni *et al.* (1997) estimated somaclonal variation in axillary branching propagation and indirect SE by RAPD fingerprinting. The plantlets derived from axillary branching propagation exhibited no variation for any of the 75 RAPD markers obtained from eight different decamers whereas RAPD finger prints of 9 of 39 plantlets regenerated by indirect embryogenesis differed from that of the donor for at least one primer and one polymorphic amplification product.

In the study, we scored a maximum of 11 alleles for OPE-01 primer implying maximum polymorphism. Piccioni *et al.* (1997) generated 19 new RAPD markers from 8 primers that were not found in the donor plant fingerprints. Most of the somaclonal variation displayed one to five polymorphic bands. While this study indicated all somaclones to be polymorphic. Piccioni *et al.* (1997) observed only six of the nine somaclones as polymorphic with two or more primers. They also concluded that axillary branching propagation was a safer cloning technique while determining RAPD markers as efficient tools for the early detection of somaclonal variants in tissue culture of alfalfa.

Most of the studies on markers reported so far have included natural propagations (Brummer *et al.*, 1995; Barcaccia *et al.*, 1994; Kiss *et al.*, 1997; Gherardi *et al.*, 1998). In most of the cases, high level of genetic variation was found within and between populations while most of the loci were highly polymorphic where as in very few population, specific polymorphisms were identified. Similar results were obtained in our study on somaclones also. Linkage maps have been constructed

using RAPD and RFLP of diploid genotypes (Veronesi *et al.*, 1997) where genetic markers were used for germplasm characterization, marker assisted selection and varietal classification.

Dendrogram analysis :

Phylogenetic relationship with genetic relatedness was studied by dendrogram analysis of polymorphic RAPD marker, among 5 somaclones and their parent. In contrast to dendrogram analysis using isozyme loci, dendrogram from RAPD analysis has revealed a single group implying closeness of somaclones to their parent. This has indicated the superiority of isozyme loci in distinguishing somaclones over RAPD markers. Tavoletti *et al.* (1998) also used cluster analysis and principal coordinate analysis to characterize genetic diversity in 3 diploid populations of *M.sativa* complex. They also concluded that the high level of genetic diversity could be efficiently applied in genetic mapping of five mutants.

Comparative analysis of two dendrogram:

While two dendrogram generated separately from isozyme and RAPD have similarly implying a trend in genetic relatedness, isozyme seemed to be distinguishing somaclones more authentically than RAPD markers which were randomly selected. Both the dendrograms indicated S5 somaclone most distant from parent while S1 and S3 were the most closest to parent according to isozyme and RAPD analysis, respectively. Major difference in the results appeared to be in the number of clusters that were three and one for isozymes and RAPD analysis respectively. Order of and genetic relatedness of somaclones appeared to be similar between two dendrograms. The minor difference among dendrograms could be ascribed to the area of genome sampled through RAPD and isozymes. Nevertheless, dendrogram generated from isozyme could be considered for breeding purposes in view of large number of loci studied.

5.2.4. Chemical analysis:

Nutritional composition of germplasm in any species is very useful to have the first hand knowledge of genetic variability for subsequent development of nutritionally superior/ better cultivars of that particular crop/species. Nitrogen content together with the cell wall contents are the most important factor to the voluntary intake (Van Soest, 1994).

Crude protein content in the germplasm of lucerne under study varied between 24.0-34.6 %. Concentration of cell wall fractions (NDF, ADF, Cellulose and lignin) ranged from 20.4-33.7, 14.2-23.0, 10.8-18.6 and 3.7-5.2 % amongst the evaluated /somaclones of lucerne germplasm. Panwar *et al.* (1992) reported lower CP and higher fiber contents in the two tested varieties of lucerne (T-9 and LH-84). CP contents was 15.66-16.17 %, while NDF and ADF contents were 67.65-70.05 % and 46.47-53.47 %, respectively in these varieties. On the other hand mean contents of different lucerne cultivars for CP, NDF, ADF, cellulose, hemicellulose and lignin (20.30, 43.92, 34.90, 24.41, 9.02 and 9.37 %) reported by Tewatia *et al.* (1998) are differed than the present findings. Higher CP contents and lower fiber fractions in the somaclones of present study may be attributed either to the differences in the stage of crop harvested for evaluation or due to occurrence of somaclonal variation. Age of crop, environment, genetic variability and nutrition (fertilizer) are same of the factors affecting CP and cell wall contents of a crop. Tewatia *et al.* (1998) observed lower CP (17.10 %) and higher crude fiber (26.30%) in the hay of lucerne harvested at the mature stage of growth. As crop matured there was a decrease in cell contents (CP, chlorophyll, sugar etc.) and increase in fiber (NDF, ADF, cellulose and lignin) accumulation. Mahanta *et al.* (1999) evaluated diploid and tetraploid lines of berseem and reported that wide variability existed in different lines in respect of quality traits (CP, NDF, ADF, cellulose and lignin) which confirmed that genetic differences occurs in germplasm of different forage species.

SUMMARY

5. SUMMARY

The results of various experiment conducted in the present study have been categorized under two major groups. The first major group has been comprised of the experiments on optimization of induction, quality and growth of the callus and regeneration of plantlets from different explants in eight genotypes. LLC-3, LLC-9 IL-75, A-3, IG-1212, AL-95-12, C-10 and Anand-2 of *M.sativa*. The second major group consisted of experiments on evaluation of *in vitro* regenerated plants, the somaclones, survived in field conditions in comparison with the respective parent plant materials for various morphological traits, isozyme RAPD and the chemical analyses.

For callus induction, the effect of different basal media (SH, MS and Blaydes) from hypocotyl, epicotyl and cotyledon explants revealed that MS medium performed better than the other two media for callus induction, frequency, callus colour and callus texture.

Effect of explant on callus induction and callus quality was distinctly observed. The cotyledon explant exhibited maximum callus induction frequency followed by hypocotyl explant. For callus colour and callus texture also, the cotyledon explant was found best followed by epicotyl explant on MS medium.

Genotypic effect was found prevalent for callus induction and callus quality. Among all the eight genotypes, IG-1212 followed by AL-95-12 responded best for callus induction frequency, callus colour and callus texture.

Among the various combinations of growth regulators with SH and MS basal media, the MS medium with 2.0 mg/l 2,4-D, 1.0 mg/l NAA and 0.2 mg/l BAP showed maximum callus induction followed by the some combinations of growth regulators with SH medium for all the explants. Inclusion of 2,4-D in the medium was found

useful for enhancing callus induction from various explants in all the genotypes of lucerne.

Callus growth rate in terms of fresh and dry callus weights and per cent dry matter content accumulation in the calli during various passages of subcultures indicated that there was a marked variation in these parameters as affected by different genotypes. In general, all the genotypes showed steady growth of the callus up to 40-60 days and there was an initial increase in the dry matter content up to 40 days followed by gradual decrease till 80 days of the callus growth. The optimum callus growth was observed between 20 and 40 days of callus culture.

The calli use for various experiments on morphogenetic differentiation and regeneration were taken from the stages of 20-40 days in culture from all the three explants of all the 8 genotypes under study. The studies on morphogenetic differentiation of the calli and regeneration of plants were carried out with the best quality of the calli obtained from different explants of the 8 genotypes following two pathways-1) Morphogenetic response of calli directly on regeneration media, and 2) Morphogenetic response of calli on the sequence of regeneration media following auxin shock treatment.

Different 96 MS and SH basal based regeneration media combinations having different auxins and cytokinins and without auxin and cytokinin were used for morphogenetic differentiation of calli. Different morphogenetic responses of callus differentiation, such as, 1) different types of callus quality ranging from white, yellow, green, to somewhat brown callus in colours and nodular to friable in texture, 2) differentiating types of calli of different colour ranging from white, yellow to green and 3) somatic embryos of various stages of somatic embryogenesis were observed.

The development of somatic embryos was observed only on MS basal medium without any growth regulators from the calli of cotyledon explant of LLC-3 genotype.

In the second pathway of regeneration, different regeneration media sequences, namely, SHKI, MSKI, SHPKI and MSPKI were used for morphogenetic response of calli following auxin sock treatment. While SHKI, MSKI and SHPKI regeneration media sequences resulted to various types of morphogenetic calli, only it was only the MSPKI regeneration media sequence on which morphogenetic differentiation of the callus leading to shoot bud organogenesis and shoot formation occurred. Incorporation of proline, in MS medium based MSPKI sequence of media was found to be necessary for regeneration of shoot buds. Maximum efficiency of morphogenetic differentiation of shoot buds was observed from the cotyledon derived calli of LLC-3 genotype followed by epicotyl derived calli of IL-75 genotype. Shoot bud organogenesis was also observed from the calli of hypocotyl explant of the genotype IL-75 and from the calli of cotyledon explant of A-3 genotype on MSPKI regeneration media sequence.

The microscopic examination revealed that regenerating calli were composed of compact and round shaped cells of comparatively small and uniform size with densely staining cytoplasm as opposed to the vitrious and other types of nonregenerating calli which were composed of loose mass of cells having various shapes and sizes ranging from small ovoid to large vesicular, tubular or syphonous cells with feebly stainable cytoplasm and obscure nucleus.

On histological examination, the developing somatic embryos exhibited bipolar meristematic structures with a plumule and radical region at distal and proximal ends respectively and the developing shoot buds were observed as unipolar meristematic outgrowths which assumed the structure of vegetative shoot buds having clearly discernible tunica-corpus organization with laterally subtending leaf primordia.

The regenerated shoots were like shoot stumps having very little or no roots at the base. Rhizogenesis was induced on various root induction media. The maximum rooting response was observed in the genotype LLC-3 followed by IL -75. The

maximum root induction was observed on SH medium containing 0.5 mg/l kinetin, 2.0 mg/l NAA, 5.0 mg/l IAA and 3.0 g/l charcoal. Addition of charcoal enhanced the rooting response.

The plantlets with well developed shoots and roots were subjected to hardening for their acclimatization from culture to field conditions. Successful hardening and acclimatization of the *in vitro* regenerated plants and their survival could be possible only in genotype LLC-3. Out of 78 regenerated plants, only 42 plants, regenerated from cotyledon explant from LLC-3 genotype, could be survived in the field.

The somaclones established in the field exhibited bi-directional variation for all the morphological traits, studied. The mean performance of the somaclones deviated considerably for most of the traits as compared to the parent. Overall performance of somaclones was found better over to that of the parent with respect to the number of nodes and internodes, stem girth and flower size.

Maximum number of superior somaclones was recorded for stem girth followed by number of nodes and length of internodes and the maximum number of somaclones which performed lower to that of the parent was found for stem dry weight followed by leaf dry weight and leaf/stem fresh and dry weights.

The range of bi-directional variation was substantial in case of plant height, branches per plant, fresh and dry weights of leaf and stem, total green and dry weights, per cent dry matter content and flower size.

Somaclonal variation frequency varied considerably for various morphological characters. While for stem girth and dry weight stem, most of the somaclones deviated significantly from the parent, the minimum variation was noticed with regard to per cent dry matter content. Very high frequency of somaclonal variation was also noted in case of length of internode, leaf/stem fresh weight and the number of nodes.

Somaclones were characterized using isozymes of the six enzymes. All the isozymes clearly indicated variation among the somaclones. Zymogram analysis showed that all the somaclones differed mostly from the parent for PGI, PGM, G6PDH, AAT, SOD and Esterase. Maximum number of bands were found in G6PDH, SOD and Esterase. Dendrogram based on isozyme analysis showed that somaclones S1 and S2 were genetically closer to the parent where as the somaclone S5 was the most distant.

Somaclones were further characterized using RAPD polymorphism. Variation among somaclones was observed in the DNA banding patterns in case of primers such as OPJ-01, OPJ-02, OPJ-05, OPJ-06, OPM-04 and OPE-01. This clearly indicated somaclonal variation. Maximum polymorphism was recorded from OPE-01. Maximum number of bands was found in OPJ-01 and OPE-01 followed by OPJ-05, OPJ-06 and OPE-01. Dendrogram based on RAPD analysis showed somaclone number 3 as the closest to the parent while somaclone number 5 was genetically the most distant from the parent.

Based on the quality parameters for chemical analysis of the somaclones, the somaclone S5 was found better in CP content than the parent while the parent was having more CP content than the other somaclones. The cell wall contents, in general, were relatively lower in the parent but lignin content was found less in 3 somaclones S5, S1 and S6, least being in somaclone S5. The lowest levels of the cellulose and ADF and lignin contents were recorded in the somaclone S5 has found best followed by somaclone S1 both being superior over the parent. The somaclone S5 was rated as the best as per the over all assessment of chemical analysis for forage quality parameters and availability index.

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